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13. ABSTRACT (Maximum 200 Words)

Two major independent barriers against the successful therapy of breast cancer are mutation of the tumor suppressor p53 gene and overexpression of the c-erbB-2/neu (HER-2/neu) gene. However, there is little or no information on how, if at all, these molecular defects together influence therapeutic outcome. Of further concern is the absence of any therapeutic agents that could be used against both defects. The present research project was proposed to address these limitations.

The results from this project indicate that both p53 (wild-type and mutant) and overexpression of c-erbB-2/neu lead to cisplatin resistance, and that the resistance due to wild-type p53 and c-erbB-2/neu overexpression can be circumvented by DACH-acetato-Pt. The fact that under certain cellular context, wild-type p53 can lead to substantially greater resistance to an antitumor agent is a novel finding that may have greater limitations in the treatment of breast cancer. The data further indicate that overexpression of c-erbB-2/neu can interfere with p53 regulation when the DNA damaging agent is cisplatin, but there is no effect on regulation when the damage is induced by DACH-acetato-Pt. This suggests that the novel compound may have clinical utility in the treatment of breast cancer which overexpresses c-erbB2/neu.

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Introduction

Two of the major barriers against the successful therapy of breast cancer are mutation of the tumor suppressor p53 gene and overexpression of the c-erbB-2/neu (HER-2/neu) gene. These conclusions have been drawn from many studies reported over the last decade for each of these two genetic defects. However, the possibility of interactions between these two genes in the treatment outcome with therapeutic antitumor agents are limited, and have led to conclusions that are inconsistent. The end result is that treatment decisions are made in absence of any objective information to guide the design of the regimen. Of further concern is the absence of any therapeutic agents that could be used under such circumstances. The present research project was proposed to address these limitations.

During the course of the studies supported by the US Army DoD Breast Cancer Program, we have obtained evidence to support the concept that drug resistance can arise by dysfunctional wild-type p53, but that DACH-acetato-Pt (also referred to as DAP in this Report) circumvents this resistance by recativating p53, which then over-rides the effects of c-erbB-2/neu overexpression. We have used transfection studies and have gained data from biochemical pharmacologic and molecular biologic investigations to demonstrate that effective treatment of cancers overexpressing c-erbB2/neu is a viable clinical proposition. In conclusion, the studies have identified pathways that become dysfunctional in breast cancers and affect drug resistance, and how this resistance may be circumvented.

Body of Report

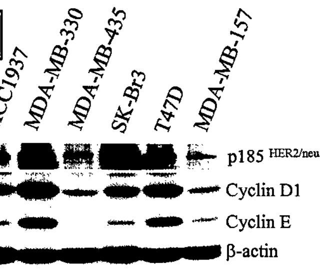
The course of this DoD-supported breast cancer project, we have undertaken studies defined originally under the 7 Tasks, and summarize here the outcome of the investigations.

Task 1

We identified several breast tumor models for use in the project, and characterized their status with regard to p53 and c-erbB-2/neu. We used literature information, p53 gene status using WAVE analysis, and Western immunoblots. Our conclusions are indicated in Figure 1, Table 1, and Table 2. Note that we extended the studies to include cyclin D1 and E because of the potential association of c-erbB-2/neu and these cyclins (Figure 1). It appears that overexpression of p185$^{\text{HER-2/neu}}$ (product of c-erbB-2/neu) correlates with overexpression of these cyclins. The implications for this to the PI3-K/Akt pathway is obvious, and we will attempt to include this pathway in pertinent discussions below.

An aspect of our Task 1 was to identify and confirm a breast cancer model with a characteristic of having null p53 and overexpressing c-erbB-2/neu, but this was unsuccessful. Our secondary option to select the p53-null MDA-MB-157 cell line and then sequentially transfect with p53 and/or c-erbB-2/neu gene also did not result in success. We considered the c-erbB-2/neu overexpressing SK-Br3 as a possibility, but the presence of a dominant-negative p53 in this cell line was considered as a potential impediment for assessing the effect of exogenous wild-type p53. As a proposed back-up option, we utilized the p53-null SKOV-3 cell line which overexpresses c-erbB-2/neu. Although the SKOV-3 is a cell line of ovarian origin, the etiology of this disease is similar to breast cancer and can provide significant information. We have successfully transfected the SKOV-3 cell line with a temperature-sensitive (ts) p53 vector, and selected stable clones for investigations (see below).

Fig. 1



The cell lines were characterized for their growth over a period of time, and the number of cells that were needed for seeding 96-well plates to provide a liner log-phase growth over the duration of time required for the MTT cytotoxic assay was established. This was also assessed for the temperature-sensitive SKOV-3 transfectant clones at both 37°C (non-permissive) and 32°C (temperature-permissive).

Table 1. Characteristics of breast tumor models

Model	MCF-7 par	MCF-7/neo	MCF-7/Her2-18	ZR75-1	MDA-MB-435 par	MDA-MB-435/neo	MDA-MB-435/eB1	SK-Br3
p53 status	wild-type	wild-type	wild-type	wild-type	mutant	mutant	mutant	mutant
c-erbB-2/neu overexp	low	low	high	low	no	no	high	high

par = parental cell line.

Task 2

The cytotoxic data with cisplatin and the analog DACH-acetato-Pt were determined in our panel of breast tumor models, and were examined in the light of HER-2/neu, p53, 473P-Akt and PTEN status, where known. The Akt phosphorylated at serine 473 (473P-Akt) and PTEN (encodes a phosphatase regulating 473P-Akt) were included in the study because of the potential involvement of the PI3-K/Akt pathway (see Figure 1 and Task 1). Ser-473 phophorylated Akt is associated with poor therapeutic outcome in breast cancer.

The results of our investigations are indicated in Table 2, which also indicates where known the status of the appropriate genes of interest. The IC_{50} of cisplatin did not indicate dependency on wild-type p53 (0.65-9.58 μM in wild-type p53 models vs. 0.59-7.43 μM in mutant/null models). In contrast, DACH-acetato-Pt appeared to do better against wild-type models than mutant/null p53 models (0.17-0.83 vs 1.10-1.84 μM). This is consistent with out finding in ovarian cancer models [Hagopian et al., 1999]. Interestingly, although DACH-acetato-Pt was more active than cisplatin against the wild-type p53 cell lines, as demonstrated by the cytotoxic ratios of 2.8-11.3, it was unexpectedly also more active against the mutant p53 models MB-330 and T47D, further demonstrating novelty of the molecule. It is noteworthy that the high IC_{50} of cisplatin correlated in general with a high HER-2/neu, Akt, cyclin D and/or cyclin E expression, whereas the effect of these molecular factors, if any, on analog-induced cytotoxicity appeared to be considerably less.

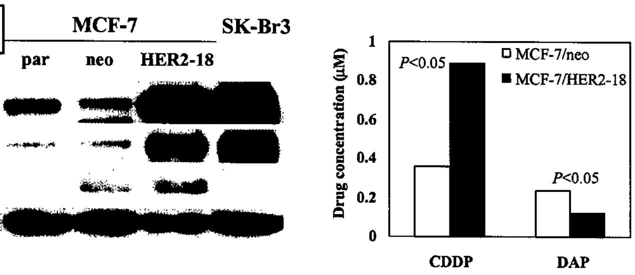
Table 2. Cytotoxicity of cisplatin and DAP in a panel of breast tumor models

Cell line	HER2	p53	473P-Akt	PTEN	IC50 value (μM)		Cisplatin/DAP ratio
					Cisplatin	DACH-ac-Pt (DAP)	
MCF-7	+	wt	low	wt	0.83 ± 0.27*	0.17 ± 0.12	4.9
ZR75-1	+++	wt	high	mu	9.58 ± 1.59	0.85 ± 0.29	11.3
HCC1937	++	wt	nk	nk	0.65 ± 0.11	0.23 ± 0.05	2.83
MDA-MB-330	++++	mu	nk	nk	7.43 ± 2.55	1.10 ± 0.44	6.75
MDA-MB-435	+	mu	nk	nk	0.84 ± 0.06	1.11 ± 0.36	0.76
SK-Br3	++++	mu	high	wt	0.59 ± 0.24	1.38 ± 0.09	0.43
T47D	+++	mu	high	nk	4.89 ± 0.30	1.84 ± 0.52	2.66
MDA-MB-157	+	null	nk	nk	0.75 ± 0.23	1.29 ± 0.49	0.58

* Mean ± SD; n=3-5. wt = wild-type; mu = mutant; nk = not known

In order to confirm the status of c-erbB-2/neu pverexpression in resistance, we utilized the MCF-7 models and MDA-MB-435 models that were transfected with the c-erbB-2/neu (Her-2/neu) gene. The MCF-7/HER2-18 model overproduces total and phosphorylated $p185^{HER-2/neu}$ proteins compared to neo and parent (par) controls, and to the same extent as endogenous levels in the positive control SK-Br3 model (Fig. 2; left panel). The

Fig. 2



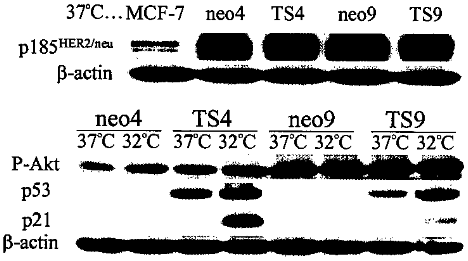
MCF-7/HER2-18 cells also show a slight increase in levels of MDM2, which is a negative regulator of p53. These changes result in a 2- to 3-fold increase in the IC_{50} concentration of cisplatin as a reflection of drug resistance (Fig. 2; right panel). In contrast, HER2-18 cells were significantly more sensitive than controls to DACH-acetato-Pt (DAP). This is an important seminal observation. The MCF-7 models harbor wild-type p53, and, therefore, the MDA-MB-435 models possessing mutant p53 were also examined for drug response (IC_{50} [μM] shown in Table 3). The interesting findings in the MCF-7 model pair were reproduced. In contrast, the MB-435/neo control cells, which were relatively resistant to cisplatin compared to MCF-7/neo cells (1.41 vs. 0.34 μM), did not show any additional resistance to cisplatin when overexpressed with HER-2/neu (1.41 vs. 1,15 μM). The increased sensitivity of MB-435/eB1 cells to DACH-acetato-Pt was modest. These results suggest that wild-type p53 may be vital for the activity of DACH-acetato-Pt to be manifested.

Table 3

Cell line	Cisplatin	DAP
MCF-7/neo	0.34 ± 0.04*	0.18 ± 0.08
MCF-7/HER2-18	0.94 ± 0.31**	0.055 ± 0.016**
MDA-MB-435/neo	1.41 ± 0.33	1.75 ± 0.41
MDA-MB-435/eB1	1.15 ± 0.12	1.31 ± 0.34**

*Mean ± SD; **$P<0.05$, vs. neo with paired *t*-test; n=3-5

Figure 3. Western Analysis of neo and TS Clones at 37°C and 32°C



However, it is clear that the mutant p53 SK-Br3 model is more sensitive to cisplatin (low IC_{50}) than would be predicted from the high expression of $p185^{HER-2/neu}$ (see Table 2). On the other hand, ZR75-1 cells have wild-type p53 and express high $p185^{HER-2/neu}$ levels, but are also substantially resistant to cisplatin (high IC_{50}). Thus, there is no clear relationship between p53 and cisplatin cytotoxicity when c-erbB2/neu is overexpressed, and it is likely that other genetic factors may play a role in modulating the IC_{50}. Conversely, the p53 may be wild-type but may be devoid of normal p53 function, as we have previously reported for ovarian tumor cells. For this reason, it was important to re-assess the role of p53 in the cytotoxicity of tumor cells overexpressing c-erbB-2/neu. The required studies were undertaken in p53-null SKOV-3 transfectants, which carry the neo or temperature-sensitive (TS) p53 gene (Figure 3). Compared to negative-control MCF-7 cells, the neo or TS clones from SKOV-3 cells overexpress c-erbB-2/neu gene. The expression of p53 is also apparent in the Western immunoblot, and its ability to transactivate $p21^{Waf1/Cip1}$ at 32°C validates the temperature-sensitive nature of the p53 (Figure 3). All clones express high levels of Ser-473 phosphorylated-Akt that are not altered at the permissive temperature of 32°C. These clones were next evaluated for sensitivity to cisplatin and DACH-acetato-Pt, and the results from three independent experiments are shown in Table 4. At 37°C, when TS clones express mutant p53, the cytotoxicity of cisplatin against TS tumor cells was either unaffected (TS9: 2.33 vs. 2.16 μM) or decreased two-fold (TS4: 0.84 vs. 1.63 μM). This increase in resistance of TS4 cells to cisplatin may reflect gain-of-function by mutant p53. The response of either TS model at the non-permissive temperature was unaffected by the presence of mutant p53 when compared to null-p53 neo cells. Compared to 37°C, the IC_{50} values at 32°C were generally increased, which is expected from thermodynamic considerations. However, the presence of wild-type p53 at the lower temperature resulted in no change in the cytotoxicity to cisplatin. Thus, wild-type p53 does not contribute to the cytotoxicity of cisplatin when cells overexpress c-erbB-2/neu. Interestingly, both TS clones displayed a significant increase in sensitivity to DACH-acetato-Pt (DAP) when

Table 4. IC_{50} (μM) of Cisplatin and DAP in neo Control and TS p53 Clones

	37 degrees		32 degrees	
clone	cisplatin	DACH-acetato-Pt	cisplatin	DACH-acetato-Pt
neo4	0.84 ± 0.24*	1.58 ± 0.29	5.94 ± 1.08	14.3 ± 3.1
TS4	1.63 ± 0.12**	2.19 ± 0.39	5.86 ± 0.82	5.91 ± 1.35**
TS4/neo4 ratio	*1.94*	*1.39*	*0.99*	*0.41*
neo9	2.33 ± 0.61	11.1 ± 1.5	4.27 ± 0.82	22.7 ± 5.6
TS9	2.16 ± 0.40	8.78 ± 2.43	3.55 ± 1.76	4.63 ± 0.24**
TS9/neo9 ratio	*0.93*	*0.79*	*0.83*	*0.20*

*Mean ± SD; **P<0.05, vs. neo with t-test; n=3

wild-type p53 is present (Table 4). These results collectively demonstrate the lack of effect of wild-type p53 on the cytotoxicity of cisplatin, and this contrasts with increased cytotoxicity of DACH-acetato-Pt in the presence of wild-type p53.

The data in this Task 2 are extremely important as they show that selected breast tumor models demonstrating high intrinsic resistance to cisplatin (ZR75-1, MDA-MB-330 and T47D) respond to DACH-acetato-Pt. Moreover, it appears that wild-type p53 does not affect the cytotoxicity of cisplatin in tumor cells overexpressing c-erbB-2/neu, but wild-type p53 in combination with DACH-acetato-Pt can circumvent cisplatin resistance. We speculate that in the case of mutant p53 models MDA-MB-330 and T47D, DACH-acetato-Pt overcomes the barrier due to HER-2 signaling, but requires wild-type p53 for greater effectiveness. The observation of reversal of cisplatin resistance by DACH-acetato-Pt in ZR75-1 cells, which is well-known for harboring mutant PTEN, is particularly noteworthy, and suggests that the high resistance to cisplatin may be due to a combination of HER-2/neu overexpression and PTEN mutation. It is noteworthy that cisplatin resistance correlated in general with a high c-erbB-2/neu, Akt, cyclin D and/or cyclin E expression, whereas the effect of these molecular factors, if any, on DAP-induced cytotoxicity appeared to be considerably less. We, therefore, conclude that either DACH-acetato-Pt acts independent of the c-erbB-2/neu/PI3-K/Akt network or suppresses this network.

Although our proposal in Task 2 was also to extend the studies to in vivo models, this proved difficult as the transfected tumor cells, essential for proof of concept, did not grow in nude mice.

Task 3

That DACH-acetato-Pt circumvents resistance in breast tumor cells can be due to restoration of wild-type p53, possibly through suppression of the c-erbB-2/neu/PI3-K/Akt network as suggested above, or may be simply attributable to a more favorable biochemical pharmacology, which permits greater intracellular accumulation of the analog and resultant greater DNA adduct formation. To confirm or eliminate this possibility, we conducted experiments to determine, drug uptake, adduct formation, and DNA damage tolerance in MCF-7 and SKOV-3 transfectant cell lines.

The results with the MCF-7 models are shown in Table 5, and demonstrate that drug uptake and DNA adduct formation for each platinum agent (100 μM x 2 hr) were similar in control and MCF-7/HER2-18 cells. It should be noted that drug uptake and adduct formation for

Table 5. Biochemical Pharmacology of Cisplatin and DAP in MCF-7 neo and HER2-18 Clones (*Mean ± SD; **P<0.05 vs. neo, using t-test; N=3-6)

	Cellular uptake (ng platinum/mg protein)		DNA adduct formation (ng platinum/mg DNA)		DNA-damage tolerance (ng platinum/mg DNA)	
Cell line	Cisplatin	DACH-acetato-Pt	Cisplatin	DACH-acetato-Pt	Cisplatin	DACH-acetato-Pt
MCF-7/neo	145.4±21.4*	55.6±9.1	59.8±17.7	15.1±4.5	0.20±0.03	0.027±0.012
MCF-7/HER2-18	149.9±42.5	71.7±17.6	66.5±13.8	17.9±3.9	0.63±0.21**	0.010±0.003**

Table 6. Biochemical Pharmacology of Cisplatin and DAP in neo and TS Clones from SKOV-3 Cells

	37 degrees		32 degrees	
clone	cisplatin	DACH-acetato-Pt	cisplatin	DACH-acetato-Pt
Platinum uptake (ng Pt/mg protein)				
neo4	81.8±33.8*	31.8±12.1	46.4±21.4	19.2±5.4
TS4	81.5±32.3	31.8±13.7	45.1±21.1	19.0±6.8
DNA adducts (ng Pt/mg DNA)				
neo4	39.8±12.8	7.7±1.4	20.8±10.7	7.9±2.5
TS4	30.1±10.6	7.1±2.1	19.7±13.4	8.6±3.2
DNA damage tolerance (ng Pt/mg DNA at IC$_{50}$)				
neo4	0.33±0.09	0.12±0.02	1.24±0.22	1.13±0.24
TS4	0.49±0.03**	0.16±0.03	1.15±0.16	0.51±0.12**

*Mean ± SD; ***P*<0.05, vs. neo4 with *t*-test, n=3-4

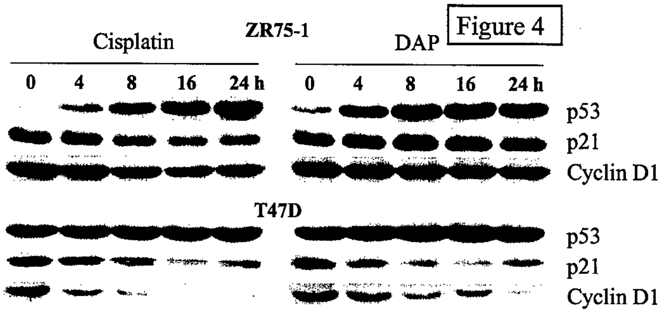
DACH-acetato-Pt were substantially lower than those of cisplatin. Thus, DNA damage tolerance was greater for cisplatin in HER2-18 cells compared to controls, but this tolerance was significantly lower with DACH-acetato-Pt as compared to controls. For further confirmation, we also examined uptake for 2 h at 20 μM drug concentration and determined DNA damage tolerance in neo4 and TS4 clones derived from SKOV-3 cells at both 37°C (when p53 is mutant) and 32°C (when p53 is wild-type). The results, shown in Table 6, provide evidence that the greater cytotoxicity by DACH-acetato-Pt was not due to increased drug uptake or increased level of DNA adducts formed. Indeed, uptake and adducts were also lower with DACH-acetato-Pt (DAP) than cisplatin in neo4 control and TS4 cells. Moreover, drug uptake and adducts formed were similar in neo4 and TS4 clones for each of the two agents, and indeed levels were lower at 32°C than at 37°C due to thermodynamic effects. These combined results strongly suggest a molecular component responsible for the increased cytotoxicity of DACH-acetato-Pt in drug resistant tumor cells.

Task 4

The goal to assess p185$^{\text{HER-2/neu}}$ as total and phosphorylated levels was achieved, and presented above in Figures 1-3. The activation of p185$^{\text{HER-2/neu}}$ through phosphorylation can stimulate the SHC-GRB2-SOS/Ras/ERK and PI3-K/Akt pathways. Since the results on Akt, cyclin D1 and cyclin E presented in Figure 1 and Table 2 indicate that the PI3-K/Akt pathway is the more important, we abandoned our efforts to pursue studies with Grb2 clones. Instead, we placed some focus on the PI3-K/Akt pathway.

Since the PI3-K/Akt can affect a number of downstream genes, including p21Waf1/Cip1 and cycline D1, we exposed c-erbB-2/neu and cyclin D1 overexpressing ZR75-1 (wild-type p53) and T47D (mutant p53) cells to cisplatin and DACH-acetato-Pt (20 μM), washed out the drug after 2 hr, and examined p53, p21 and cyclin D1 levels by immunoblot 24 hr later. These cell lines were selected also because of their greater sensitivity to DACH-acetato-Pt than cisplatin. The immunoblots from the study are shown in Figure 4. In ZR75-1 cells, both cisplatin and DACH-acetato-Pt (DAP) rapidly induce the wild-type p53, but the mutant p53 in T47D was unaffected by either agent. Irrespective of the effect of cisplatin on p53 in the two tumor models, both p21 and cyclin D1 were downregulated in each cell line. DACH-acetato-Pt, on the other hand, had minimal effect on p21 and cyclin D1 in wild-type p53 ZR75-1 cells but downregulated these two gene products in the mutant p53 T47D model. Although these results do not explain the greater cytotoxicity of DACH-acetato-Pt in these two tumor models, they do suggest that both platinum agents can downregulate the PI3-K/Akt pathway, but that an additional factor is involved in enhancing the sensitivity of the tumor cells to the platinum analog. These results, therefore, suggest a element of complexity involved in the mechanism of action of DACH-acetato-Pt, and its ability to circumvent drug resistance.

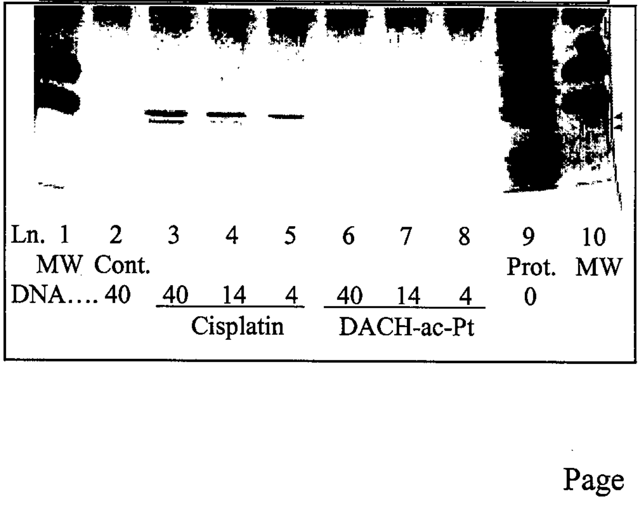
Figure 4



Task 5

Differential recognition of DNA adducts may be an important factor in distinguishing cisplatin from DACH-acetato-Pt, and this was examined by SDS-PAGE using the damaged-DNA affinity assay. In this assay, DNA damaged by cisplatin or DACH-acetato-Pt was mixed with nuclear extract, and the protein "pulled down" by the DNA was separated on the gel and silver-stained. The results are shown in Figure 5. Lanes 3-5 demonstrate that cisplatin-induced DNA adducts is recognized by HMG1 (upper of the bouble band) and HMG2 (lower band), and the levels stained were proportional to the amount of damaged DNA used in the assay (4-40 μg). In contrast, the adduct of DACH-acetato-Pt was not recognized by HMG1 or HMG2. These data indicate the differential recognition of DNA adducts may be responsible for initiating signal transduction process along independent pathways for the two platinum agents. Efforts to identify proteins that may recognize DNA damaged by DACH-acetato-Pt were unsuccessful primarily because the silver-staining does not have adequate sensitivity. Similar limitations were seen with DNA unwinding assay.

Fig. 5. Recognition of Platinum Adducts



Tasks 6 and 7

The effect of c-erbB-2/neu on p53 induction and transactivation function in tumor cells harboring wild-type p53 was the interest in Task 6, and the regulation of p53 through p53 phosphorylation was an aspect of Task 7. Since the two Tasks are better discussed together, we have combined the results of the two Tasks.

The induction, transactivation function, and post-translational regulation of p53 was evaluated in the neo and c-erbB-2/neu overexpressing MCF-7 tumor cells. Induction of total p53 was seen to be dependent of dose following a 2-h drug exposure (Figure 6) and with time following a 2-h exposure to 20 μM drug concentration (Figure 7). Inductions of phosphorylated forms of p53 ($p53^{ser-15}$ and $p53^{ser-392}$) were also observed with cisplatin. DACH-acetato-Pt, in contrast, also induced $p53^{ser-15}$ but to a lesser extent. This analog, however, was a very poor inducer of $p53^{ser-392}$. The transactivation of p21 and mdm2 is evident from the Western immunoblots, but the extent is consistent with the levels of p53 induced. However, it is clear that increased levels of $p185^{HER-2/neu}$ attenuates induction of total p53 and $p53^{ser-15}$ by cisplatin but no difference was apparent between neo and c-erbB-2/neu overexpressing cells exposed to DACH-acetato-Pt.

These results suggest that cisplatin and DACH-acetato-Pt activate independent signal transduction pathways which regulate p53, and that $p185^{HER-2/neu}$ only impinges on the pathway activated by cisplatin. There were no differences between the drugs on phosphorylation at serine-18 and serine-20 (data not shown). Attempts to establish a proteomic approach to detecting differences in post-translational modification at other sites of p53 were unfortunately abruptly ended by Tropical Storm Alison, which affected the collaboration with Baylor College of Medicine on this project due to the devastation experienced. Attempts to re-establish the collaboration were unsuccessful due to changes in priority at Baylor. The phosphopeptide mapping approach proved unsuccessful due to low sensitivity of detection.

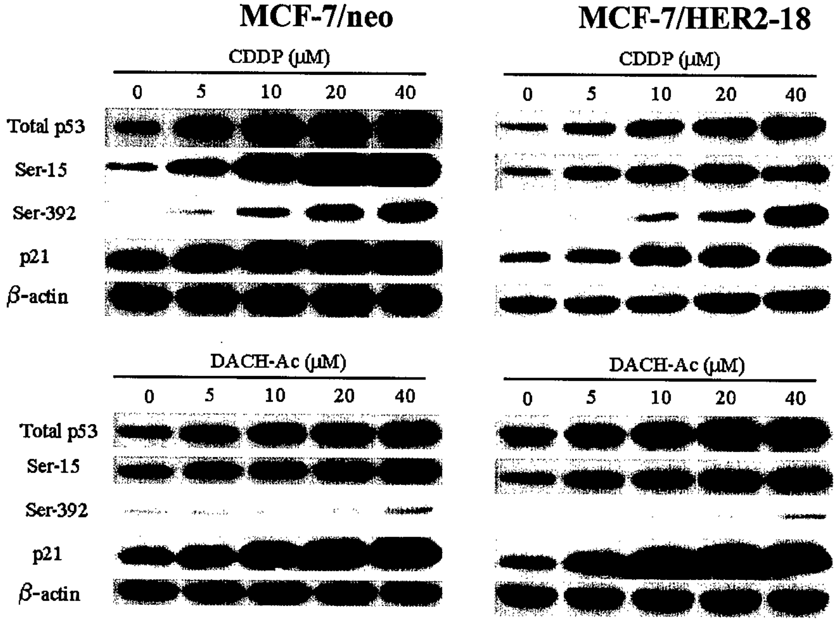


Figure 6. Concentration-dependent induction of p53 and associated proteins

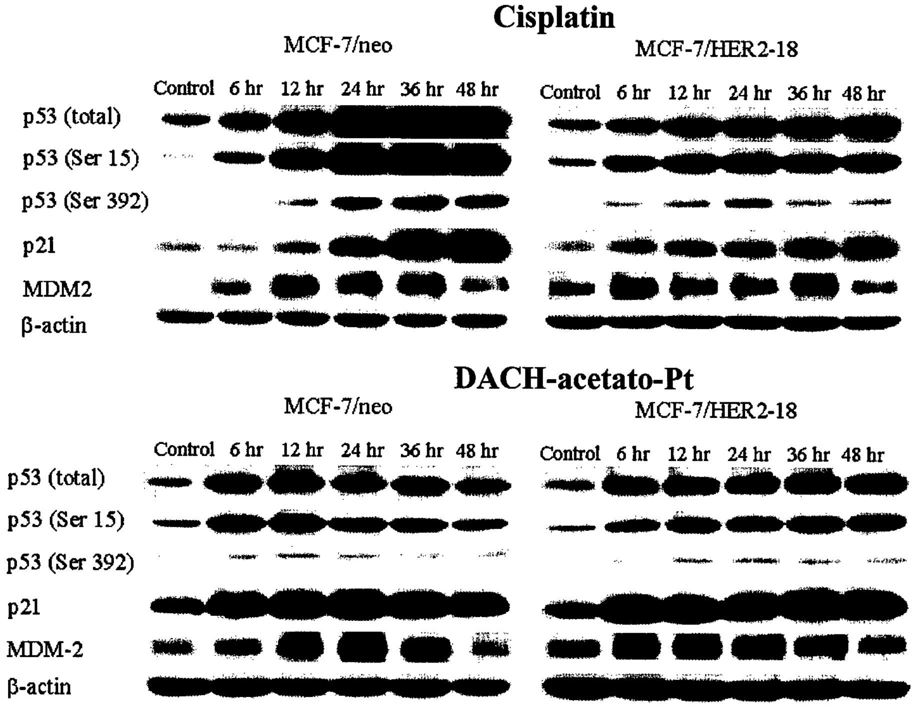

Cisplatin
MCF-7/neo
MCF-7/HER2-18
Control 6 hr 12 hr 24 hr 36 hr 48 hr
Control 6 hr 12 hr 24 hr 36 hr 48 hr
p53 (total)
p53 (Ser 15)
p53 (Ser 392)
p21
MDM2
β-actin
DACH-acetato-Pt
MCF-7/neo
MCF-7/HER2-18
Control 6 hr 12 hr 24 hr 36 hr 48 hr
Control 6 hr 12 hr 24 hr 36 hr 48 hr
p53 (total)
p53 (Ser 15)
p53 (Ser 392)
p21
MDM-2
β-actin

Figure 7. Time-dependent induction of p53 and associated proteins

In order to assess if phosphorylation involved a PI3-K kinase, we designed experiments to monitor ser-15 phosphorylation in MCF-7 cells by the platinum agents in the presence of the kinase inhibitor wortmannin. The cells were pretreated with wortmannin (1, 3 or 10 μM) for 2h, then exposed for 2h to 20 μM CDDP or DACH-Ac-Pt in the presence of wortmannin, and

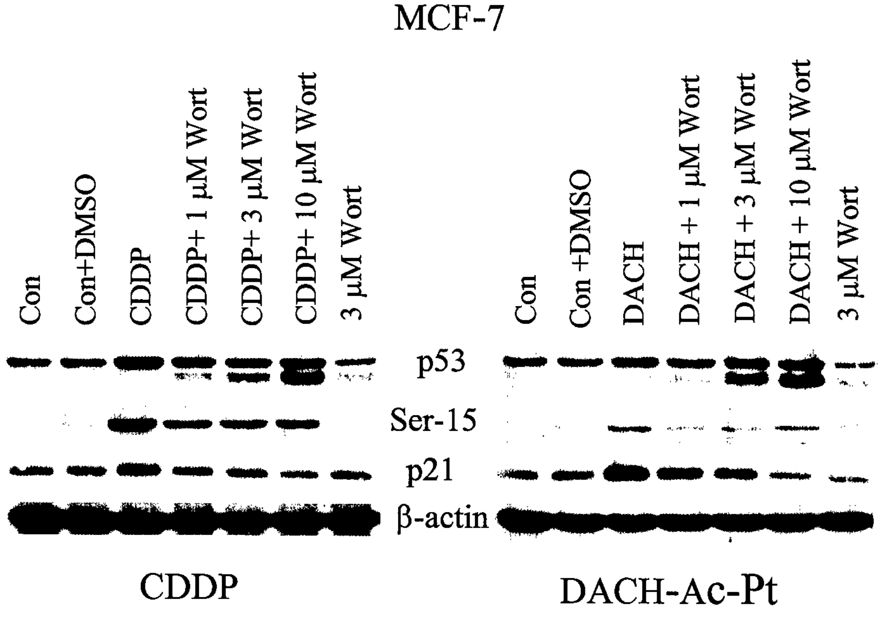
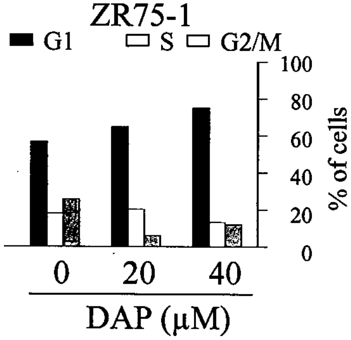

MCF-7
Con
Con+DMSO
CDDP
CDDP+ 1 μM Wort
CDDP+ 3 μM Wort
CDDP+ 10 μM Wort
3 μM Wort
Con
Con +DMSO
DACH
DACH + 1 μM Wort
DACH + 3 μM Wort
DACH + 10 μM Wort
3 μM Wort
p53
Ser-15
p21
β-actin
CDDP
DACH-Ac-Pt

Figure 8. Effect of wortmannin on Ser-15 p53 phospphorylation

treatment with wortmannin alone continued for an additional 20 hr. Following the final exposure period to wortmannin, the cells were harvested for western analysis. DMSO, the solvent for dissolving wortmannin, was used as a control. The results are shown in Figure 8. The induced Ser-15 phosphorylation by the platinum compounds in MCF-7 cells was not completely inhibited by wortmannin. Although inhibition of induced p21$^{WAF1/CIP1}$ levels by wortmannin was apparent, the basal levels of p21$^{WAF1/CIP1}$ were not affected by the inhibitor. Thus, wortmannin inhibited only the increased levels of p21$^{WAF1/CIP1}$, which presumably occurred as a result of p53 activation. Wortmannin did not cause any changes in Ser-392 phosphorylation in these cells (data not shown). These results demonstrate that members of PI3K family are involved in phosphorylating and functionally activating p53 in response to DNA damage by both CDDP and DACH-Ac-Pt.

Since flavopiridol (a cell cycle inhibitor), synergizes with LY294002 (a PI3-K inhibitor), it is reasonable to consider that DACH-acetato-Pt may demonstrate effectiveness because of its specific effects on the cell cycle. We have previously demonstrated that DACH-acetato-Pt selectively inhibits CDK4/cyclin D and CDK2/cyclin E kinase activities to induce G1 phase arrest [Kuang et al., 2001]. Cisplatin, as has been reported, induces transient S-phase arrest, followed by G2 phase arrest (data not presented). It was of interest to examine if overexpression of c-erbB-2/neu may modulate cell cycle effects of DACH-acetato-Pt. This was examined in ZR75-1 cells exposed to DACH-acetato-Pt (0-40 μM) for 2 hr, and exposing cells to FACS analysis 48 hr later. The results in Figure 9 demonstrate that G1 phase arrest by the platinum analog was unaffected by c-erbB-2/neu overexpression (Figure 9). Thus, the cyclin D1 and E overexpression mediated by c-erbB-2/neu overexpression does not hamper the cell cycle effects of DACH-acetato-Pt.

Figure 9



Key Research Accomplishments

- Overexpression of c-erbB-2/neu increases resistance to cisplatin independent of p53 status, but increases sensitivity to the analog DACH-acetato-Pt either moderately when p53 is mutant or substantially when p53 is wild-type.

- Resistance to cisplatin by c-erbB-2/neu overexpression is not due to a decrease in drug uptake or a reduction in DNA adducts formed. Conversely, the increase in sensitivity to DACH-acetato-Pt was not due corresponding increases in intracellular drug or adduct levels. In fact, the ability of cells to tolerate higher adduct levels is an index of reduced apoptotic activity, and was the main reason for resistance to cisplatin, whereas cells sensitive to the analog demonstrated reduced tolerance to adducts.

- The differential effects of p185$^{HER\text{-}2/neu}$, the product of c-erbB2/neu gene, on cytotoxicity of cisplatin and DACH-acetato-Pt appears to correlate with differences in regulation of p53, particularly at serine-392, and the differential effect on the p185-mediated Akt survival pathway. The role of p21 in combination with wild-type p53 function or cerbB2/neu overexpression was of a prognostic value in the cytotoxicity of the analog. This is consistent with a few reports that p21 may play a role in apoptosis under certain conditions [Yang et al., 2003].

- It is very likely that drug resistance by c-erbB-2/neu overexpression is mediated through the PI3-K/Akt pathway, which is downregulated by both cisplatin and DACH-acetato-Pt, but it is proposed that the analog in addition can activate p53 function through an independent pathway that mediates the apoptotic response.

Reportable Outcomes

The results arising from this project have been presented in the following form:

ABSTRACTS

Watanabe, M., Nakamura, K., Mujoo, K., Khokhar, A. R., and Siddik, Z. H. Modulation by HER2/Neu of the cytotoxicity of cisplatin and 1r,2r-Diaminocyclohehexane-dacetato-dchloro-patinum(IV)(DACH-aetato-Pt) against wild-type p53 MCF-7 breast tumor cells. Proc Am Assn Cancer Res 42(A2284), 425. 2001.

Watanabe, M., Mujoo, K., Hennessey, P., Khokhar, A. R., and Siddik Z. H. Wild-type p53 circumvents resistance of HER2/neu-overexpressing tumor cells in a drug-dependent manner. Abst. #715 – Proc AACR-NCI-EORTC International Conference. Miami Beach, FL. Oct 29 – Nov 2, 2001.

Watanabe, M., Mujoo, K., Khokhar, A. R., and Siddik, Z. H. Circumventing mechanisms of drug resistance in breast tumor cells overexpressing HER2/neu. Abstract # P55-18 – Proc. Era of Hope DoD BCRP Meeting, Orlando, FL; September 25-28, 2002.

MANUSCRIPTS: PUBLISHED/IN PRESS

Siddik, Z.H. Mechanism of action of cancer chemotherapeutic agents: platinum complexes and other DNA-interactive agents. In: The Cancer Handbook (Alison, M.R., ed.), pp. 1295-1311, Nature Publishing Group, London, 2002.

Siddik, Z. H. Cisplatin: mode of cytotoxic action and molecular basis of resistance. Oncogene, in press.

Mujoo, K., Watanabe, M., Nakamura, J., Khokhar, A. R. and Siddik, Z. H. Status of p53 Phosphorylation and Function in Sensitive and Resistant Human Cancer Models Exposed to Platinum-based DNA Damaging Agents. J. Cancer Res Clin Oncol, in press.

MANUSCRIPTS: SUBMITTED

Watanabe, M., Mujoo, K., Khokhar, A. R., and Siddik, Z. H. Differential Modulation by HER2/neu of the Cytotoxicity of Cisplatin and 1R,2R-Diaminocyclohexane-diacetato-dichloro-Platinum (IV) Against Wild-Type and mutant p53 Breast Tumor Cells. Oncogene.

MANUSCRIPTS: IN PREPARATION:

Watanabe, M., Hennessey, P., Mujoo, K., Khokhar, A. R., and Siddik, Z. H. A novel analog 1R,2R-Diaminocyclohexane-diacetato-dichloro-Pt (IV) circumvents cisplatin resistance induced by upregulation of p21$^{waf1/cip1}$ in breast cancer cell lines. In preparation.

Watanabe, M., Mujoo, K., Khokhar, A. R., and Siddik, Z. H. Wild-type p53 reduces DNA-damage tolerance of HER2/neu-overexpressing ovarian cancer cells in a drug-dependent manner. In preparation.

Conclusions

We have demonstrated that alteration in pathways involving p53 (wild-type or mutant) and overexpression of c-erbB-2/neu lead to cisplatin resistance, and suggests that c-erbB2/neu has a dominant survival effect. This was confirmed by following the phosphorylation status of the p185$^{HER-2/neu}$ and Akt proteins following cisplatin treatment. However, cisplatin resistance is circumvented by the platinum analog DACH-acetato-Pt, and this is associated with p21 expression and a lack of a durable effect on phosphorylated-p185$^{HER-2/neu}$ and Akt. Resistance or sensitivity was not due to changes in drug uptake or levels of adducts formed, but were correlated closely with the relative ability to tolerate DNA damage. The data indicate that overexpression of c-erbB-2/neu can interfere with p53 regulation and activates the Akt pathway when the DNA damaging agent is cisplatin, but there is no effect on activated p53 levels and the modest rise in phosphorylated Akt does not persist when the damage is induced by DACH-acetato-Pt. Indeed, wild-type p53 can over-ride the negative effect of c-erbB-2/neu overexpression when the cytotoxic agent is the platinum analog. Collectively, these data support the concept that DACH-acetato-Pt favorably modulates the PI3-K/Akt pathway to induce cytotoxicity against chemoresistant breast tumor cells that overexpress c-erbB-2/neu, and is, therefore, worthy of clinical trials against breast cancer.

PERSONNEL ASSOCIATED WITH RESEARCH EFFORT

Zahid H. Siddik, Ph.D.
Kalpana Mujoo, Ph.D.
Masayuki Watanabe, M.D.
Patrick Hennessey, B.S.

Reference List

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Kuang J, He G, Huang Z, Khokhar AR, Siddik ZH (2001) Bimodal effects of 1R,2R-diaminocyclohexane(trans-diacetato)(dichloro)platinum(IV) on cell cycle checkpoints. *Clin Cancer Res* 7: 3629-3639

Yang HL, Pan JX, Sun L, Yeung SC (2003) p21 Waf-1 (Cip-1) enhances apoptosis induced by manumycin and paclitaxel in anaplastic thyroid cancer cells. *J Clin Endocrinol Metab* 88: 763-772

Appendix

ABSTRACTS

1. Watanabe, M., Nakamura, K., Mujoo, K., Khokhar, A. R., and Siddik, Z. H. Modulation by HER2/Neu of the cytotoxicity of cisplatin and 1R,2R-Diaminocyclohehexane-dacetato-dchloro-patinum(IV)(DACH-aetato-Pt) against wild-type p53 MCF-7 breast tumor cells. Proc Am Assn Cancer Res 42(A2284), 425. 2001.

2. Watanabe, M., Mujoo, K., Hennessey, P., Khokhar, A. R., and Siddik Z. H. Wild-type p53 circumvents resistance of HER2/neu-overexpressing tumor cells in a drug-dependent manner. Abst. #715 – Proc AACR-NCI-EORTC International Conference. Miami Beach, FL. Oct 29 – Nov 2, 2001.

3. Watanabe, M., Mujoo, K., Khokhar, A. R., and Siddik, Z. H. Circumventing mechanisms of drug resistance in breast tumor cells overexpressing HER2/neu. Abstract # P55-18 – Proc. Era of Hope DoD BCRP Meeting, Orlando, FL; September 25-28, 2002.

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4. Siddik, Z.H. Mechanism of action of cancer chemotherapeutic agents: platinum complexes and other DNA-interactive agents. In: The Cancer Handbook (Alison, M.R., ed.), pp. 1295-1311, Nature Publishing Group, London, 2002.

5. Siddik, Z. H. Cisplatin: mode of cytotoxic action and molecular basis of resistance. Oncogene, in press.

6. Mujoo, K., Watanabe, M., Nakamura, J., Khokhar, A. R. and Siddik, Z. H. Status of p53 Phosphorylation and Function in Sensitive and Resistant Human Cancer Models Exposed to Platinum-based DNA Damaging Agents. J. Cancer Res Clin Oncol, in press.

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7. Watanabe, M., Mujoo, K., Khokhar, A. R., and Siddik, Z. H. Differential Modulation by HER2/neu of the Cytotoxicity of Cisplatin and 1R,2R-Diaminocyclohexane-diacetato-dichloro-Platinum (IV) Against Wild-Type and mutant p53 Breast Tumor Cells. Oncogene.

MANUSCRIPTS: IN PREPARATION:

8. Watanabe, M., Hennessey, P., Mujoo, K., Khokhar, A. R., and Siddik, Z. H. A novel analog 1R,2R-Diaminocyclohexane-diacetato-dichloro-Pt (IV) circumvents cisplatin resistance induced by upregulation of $p21^{waf1/cip1}$ in breast cancer cell lines. In preparation.

9. Watanabe, M., Mujoo, K., Khokhar, A. R., and Siddik, Z. H. Wild-type p53 reduces DNA-damage tolerance of HER2/neu-overexpressing ovarian cancer cells in a drug-dependent manner. In preparation.

Appendix #1

Modulation by HER2/neu of the Cytotoxicity of Cisplatin and 1R,2R-Diaminocyclohexane-diacetato-dichloro-platinum(IV) (DACH-Acetato-Pt) Against Wild-Type p53 MCF-7 Breast Tumor Cells. M. Watanabe, J. Nakamura, K. Mujoo, A.R. Khokhar, and Z.H. Siddik. Department of Experimental Therapeutics, The University of Texas M.D. Anderson Cancer Center, Houston, TX 77030.

Wild-type p53 facilitates drug-induced apoptosis, whereas HER2/neu (HER2) induces resistance to some antitumor agents, including cisplatin, and sensitivity to others. Therefore, the aim was to study the effect of the non-cross-resistant platinum complex DACH-acetato-Pt against MCF-7/HER2-18 (HER2-18) having wild-type p53 and stably-transfected HER2 gene, and a control isogenic MCF-7/neo (neo) cell line. Basal levels of HER2 by Western analysis were 5.4-fold greater in HER2-18 compared to neo cells, and the active phosphorylated-form of HER2 was detectable in HER2-18 cells but not in neo. The HER2-18 model was 2-fold resistant to cisplatin compared to neo (IC$_{50}$: 0.83 vs. 0.44 μM using continuous drug exposure; 18.2 vs. 9.8 μM using 2-hour exposures). In contrast, the HER2-18 cell line demonstrated significant collateral sensitivity to DACH-acetato-Pt by up to 2-fold compared to neo (IC$_{50}$: 0.12 vs. 0.24 μM - continuous exposures; 15.0 vs. 22.1 μM - 2-hour exposures). DNA damage tolerance to CDDP was significantly higher in HER2-18 (12 ng Pt/mg DNA) than in neo (5.8), whereas there was no significant difference between the two models exposed to DACH-acetato-Pt (1.9-2.0 ng Pt/mg DNA). Although wild-type p53 and p21$^{Waf1/Cip1}$ (p21) were induced in neo and HER2-18 models after treatment with cisplatin, the induction was significantly less in HER2-18 cells. On the other hand, both protein molecules were similarly induced in the two cell lines after treatment with DACH-acetato-Pt. Interestingly, p53 was phosphorylated at serine-15 in a dose-dependent fashion in neo cells treated with cisplatin, but phosphorylation was suppressed in HER2-18. With DACH-acetato-Pt, this phosphorylation was very low in both cell lines. In conclusion, overexpression of HER2 induces cisplatin resistance by suppressing p53 induction, possibly through down-regulating serine-15 phosphorylation of p53. DACH-acetato-Pt, in contrast, likely activates an independent p53-mediated apoptotic pathway that is facilitated by HER2 by an unknown mechanism. The results indicate that DACH-acetato-Pt may have utility in the management of breast tumors overexpressing HER2 against a wild-type p53 background. (U.S. Army Grant DAMD17-99-1-9269).

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WILD-TYPE P53 CIRCUMVENTS RESISTANCE OF HER2/NEU-OVEREXPRESSING TUMOR CELLS IN A DRUG-DEPENDENT MANNER.

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Wild-type p53 facilitates drug-induced apoptosis, whereas HER2/neu (HER2) induces resistance to some antitumor agents, including cisplatin. We have utilized two models to test the hypothesis that wild-type p53 will overcome drug resistance caused by overexpression of HER2. One model was derived from wild-type p53 bearing MCF-7 breast cancer cells transfected with HER2 (HER2-18). The second model was established by transfecting null-p53 SKOV-3 ovarian cancer cells overexpressing HER2 with a temperature-sensitive mutant p53 (TS). To test our hypothesis, we used cisplatin and the novel analog 1R,2R-diaminocyclohexane-diacetato-dichloro-platinum(IV) (DACH-acetato-Pt). HER2-18 cells were 2-fold resistant to cisplatin compared to neo controls (IC50: 0.83 vs. 0.44 µM; continuous drug exposure), whereas they were significantly more sensitive to DACH-acetato-Pt by up to 2-fold (IC50: 0.12 vs. 0.24 µM). DNA damage tolerance to cisplatin was significantly greater in HER2-18 cells (12 ng Pt/mg DNA) than in neo (5.8). Similarly, TS clones were significantly more sensitive to DACH-acetato-Pt compared to neo (IC50: 5.9 vs. 14.3 µM in clone 4; 4.6 vs. 22.3 µM in clone 9) at 32°C, when p53 functioned as wild-type. In contrast, the IC50 of cisplatin at 32°C was unaffected (IC50: 5.8 vs. 5.9 µM in clone 4; 3.6 vs. 4.3 µM in clone 9). DNA damage tolerance to DACH-acetato-Pt was significantly lower in clone TS4 (42.7 ng Pt/mg DNA) than in neo4 (95.1) at 32°C, while there was no significant difference in tolerance to cisplatin. Western immunoblots from the MCF-7 models revealed that in neo cells phosphorylation of p53 at serine 15 and serine 392 contributed to the induction of p53 with cisplatin, whereas a lesser extent of serine 15 and a very poor phosphorylation at serine 392 were seen with DACH-acetato-Pt. Increased levels of HER2 attenuated both the induction of p53 and its phosphorylation at these sites in response to cisplatin, but did not affect them following DACH-acetato-Pt exposure. These results suggest that independent pathways are involved in p53 activation for the two platinum agents and HER2 only impinges on the pathway activated with cisplatin. These results also indicate that an introduction of functional p53 increases sensitivity of cells overexpressing HER2 to DACH-acetato-Pt, but not to cisplatin. Furthermore, the results indicate that introduction of functional p53 in combination with DACH-acetato-Pt may be an effective combination against cells overexpressing HER2. (Supported by the U.S. Army Grant DAMD 17-99-1-9269, and NCI RO1 CA77332 and RO1 CA82361 to ZHS).

Appendix #3

Abstract # P55-18 – Proc. Era of Hope DoD BCRP Meeting, Orlando, FL; September 25-28, 2002

CIRCUMVENTING MECHANISMS OF DRUG RESISTANCE IN BREAST TUMOR CELLS OVEREXPRESSING HER2/NEU

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and Zahid H. Siddik

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Overexpression of HER2/neu (HER2) in tumor cells generally confers resistance and wild-type p53 mediates drug sensitivity to antitumor agents. However, with cisplatin, the negative effects of HER2 overexpression appear to override the positive effects of wild-type p53. We have identified a novel platinum analog, 1R,2R-diaminocyclohexane-diacetato-dichloro-platinum(IV) (DACH-acetato-Pt), that is dependent on wild-type p53 for activity and may have a potential in the treatment of breast cancer. We hypothesized that with this analog, wild-type p53 will have a dominant effect over HER2 and overcome drug resistance from overexpression of HER2. To test this hypothesis, we investigated two breast cancer transfection models: the wild-type p53 MCF-7/HER2-18 and the mutant p53 MDA-MB-435/eB1 cell line transfected with HER2. Control transfectants carried the neo gene.

The MCF-7/HER2-18 model was 2- to 3-fold resistant to cisplatin compared to neo (IC_{50}: 0.94 vs. 0.34 μM), whereas it demonstrated a significant 2- to 3-fold collateral sensitivity to DACH-acetato-Pt (IC_{50}: 0.055 vs. 0.18 μM). These effects could not be ascribed to changes in either drug uptake or DNA-adduct formation. However, cytotoxicity correlated with DNA damage tolerance. Thus, HER2-18 cells tolerated about 3-fold higher levels of cisplatin adducts, but about 3-fold lower levels of adducts induced by DACH-acetato-Pt. Western immunoblots revealed that phosphrylation of p53 at serine 15 and serine 392 contributed to the induction of p53 with cisplatin, whereas a lesser extent of serine 15 and a very poor phosphorylation at serine 392 were seen in DACH-acetato-Pt. Increased levels of HER2 attenuated these effects of cisplatin, but had no affect when DACH-acetato-Pt was used. These results suggest that independent pathways are involved in p53 activation by the two platinum agents and HER2 differentially impinges on these independent pathways. In order to examine p53-dependence, parallel studies were conducted in the mutant p53 MDA-MB-435/eBI model. The IC_{50} of cisplatin was similar in the neo and eB1 models (1.41 vs. 1.15 μM). Similar values were seen with DACH-acetato-Pt (1.75 vs. 1.31 μM) although the increased cytotoxicity (low IC_{50}) of the analog in eB1 cells was significant ($p < 0.05$). Again, cytotoxic differences induced by HER2 in the eB1 model correlated best with DNA damage tolerance. Thus, the increased resistance to cisplatin and the greater sensitivity to DACH-acetato-Pt by HER2 appear to be dependent on the presence of wild-type p53.

These results suggest that DACH-acetato-Pt has potential clinical utility against breast cancers overexpressing HER2, but the potential is much greater in the presence of pre-existing or transfected wild-type p53.

The studies were supported by the U.S. Army Medical Research Materiel Command under DAMD17-99-1-9269

Appendix #4

Chapter 1B

Mechanisms of Action of Cancer Chemotherapeutic Agents: DNA-interactive Alkylating Agents and Antitumour Platinum-based Drugs

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CONTENTS

- Introduction
- General Mechanism of Action
- Nitrogen Mustards
- Aziridines
- Alkyl Sulfonates
- Nitrosoureas
- Platinum-based Agents
- Conclusion
- Acknowledgements

INTRODUCTION

85.1 The long-term understanding of cancer growth is that it is a net result of uncontrolled multiplication of cells that out paces the rate of natural cell death within the tumour mass. The ultimate aim of the tumour is survival by overcoming the many barriers in its path. The imperative need for continual supply of nutrients to new growth areas, for instance, is met through sustained angiogenesis. Another major factor for growth and survival is limitless replicative potential, requiring continued biosynthesis of the genetic material to provide a complementary set of chromosomes in each of the daughter cells following cell division. Inhibiting DNA replication, therefore, affords a logical approach for retarding tumour growth. For this reason, DNA has become a critical target in cancer chemotherapy. Indeed, many of the antitumour agents currently in the cancer armamentarium are DNA-interactive. Among them, the DNA alkylators or cross-linkers, which includes the platinum-based drugs, are the most active available for effective cancer management.

85.2 Historically, nitrogen mustard was introduced in 1942 as the first alkylating agent to have clinical utility (Gilman and Phillips, 1946). It was an analogue of the highly toxic sulfur mustard gas, which had been used as a weapon in 1917 during the First World War and later as a therapeutic agent against squamous cell carcinoma (Adair and Bagg, 1931). The advent of nitrogen mustard was the beginning of modern cancer chemotherapy, and it spawned a series of more effective and less toxic alkylating agents that are still in use today. Five major structural classes of alkylating agents are of considerable interest: the nitrogen mustards, the aziridines, alkyl sulfonates, the nitrosoureas and the mechanistically distinct platinum-containing drugs.

GENERAL MECHANISM OF ACTION

85.3 By virtue of their high chemical reactivity, either intrinsic or acquired in a biological environment, all alkylating agents form covalent linkages with macromolecules having nucleophilic centres. They have no specificity, but the chance reaction with DNA forms the basis for the antitumour effects. Bifunctional alkylating agents form covalent bonds at two nucleophilic sites on different DNA bases to induce interstrand (between two opposite strands) and/or intrastrand (on same strand) cross-links. Such cross-links can have either a 1,2 or 1,3 configuration (**Figure 1**). Monofunctional agents have only one alkylating group and, therefore, cannot form crosslinks. The traditional alkylators interact with DNA (usually the N7 position of guanine) through an alkyl group, and this is distinct from a platinum-containing drug, which, although loosely referred to as an alkylating agent, forms covalent links between adenine and/or guanine bases via the platinum atom. Irrespective of the specific mechanisms

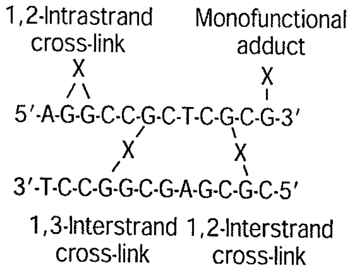
1,2-Intrastrand cross-link
Monofunctional adduct
X
X
5′-A-G-G-C-C-G-C-T-C-G-C-G-3′
X
X
3′-T-C-C-G-G-C-G-A-G-C-G-C-5′
1,3-Interstrand cross-link
1,2-Interstrand cross-link

Figure 1 Monofunctional adducts and 1,2- and 1,3-interstrand and intrastrand cross-links induced by DNA interactive agents. X = antitumour agent.

involved in the formation of adducts, the end effect of these DNA-interactive agents is to inhibit DNA replication, which in turn may affect the production of RNA and protein (Lawley and Brookes, 1965). These reactions, unfortunately, are not discriminated between normal and tumour DNA, which is a characteristic of all antitumour agents that leads to side effects and the associated low therapeutic indices. Any antitumour selectivity that is observed is dependent on the extent of covalent interactions induced by the drug that affects distortions and unwinding in DNA. Such changes in the superhelical structure are then processed as distinct signals that determine whether a cell lives or dies. When DNA is damaged, these signals inhibit cell cycle progression, which is a process that the cell activates to allow DNA repair to proceed and, thereby, prevent replication of new DNA on a damaged template or prevent damaged chromosomes to be passed on to daughter cells. Thus, as a rule, drugs that interact with DNA affect the cell cycle, and whether a cell survives or dies depends on the extent of interaction between the drug and DNA, and how rapidly the adverse effects of that interaction can be neutralized through DNA repair. Indeed, one of the mechanisms of resistance of tumour cells to alkylating and platinum agents is attributed to enhanced repair of cross-links.

85.4 In this chapter, the mechanism of action of some established DNA interactive agents will be discussed, but for many the detailed information is scant and very little is known regarding events following DNA damage. For this reason, emphasis will be placed on cisplatin, which has been studied in greater detail and, therefore, allows us to appreciate the complexity of the molecular pathway from DNA damage to cell death. Similar or overlapping pathways probably exist for the other DNA-interactive drugs.

NITROGEN MUSTARDS

85.5 Since the mid-1940s, hundreds of nitrogen mustard-based alkylating agents have been evaluated for their potential as antitumour agents. However, only a handful have found a place in medical oncology as therapeutic agents. These include nitrogen mustard (mechlorethamine), chlorambucil, melphalan, cyclophosphamide and its activated prodrug form 4-hydroperoxycyclophosphamide and ifosfamide (**Figure 2**). The common structural feature is the bischloroethyl group, which is the precursor for the activated function that predominantly alkylates the N7 of guanine, although minor alkylation reactions can also occur at other sites, including the O6 position of guanine, and N3 and N7 of adenine (Colvin *et al.*, 1999). Mechlorethamine reacts with guanine following spontaneous activation at physiological pH. The rapid rate of activation of this agent, however, is the major cause of side effects. For this reason, other members of the nitrogen mustard family are of greater interest as they have been structurally modified to regulate the generation of the active species. Cyclophosphamide, for instance, is highly stable and requires the hepatic mixed function oxidase system to activate the molecule metabolically (Sladek, 1987). Although the metabolism of cyclophosphamide is complex, the product 4-hydroxycyclophosphamide is considered the most significant. This metabolite distributes throughout the body, including the tumour where spontaneous degradation occurs to form phosphoramide mustard or nornitrogen mustard (**Figure 3**). It is useful to note that a byproduct of cyclophosphamide activation is acrolein, which is responsible for haemorrhagic cystitis as a serious side effect (Cox, 1979).

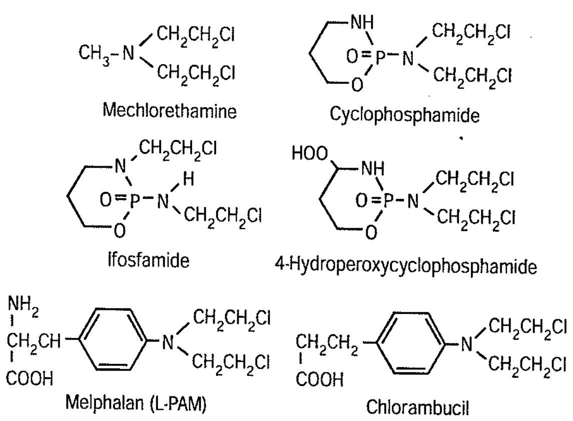

Figure 2 Structures of selected members of the nitrogen mustard family of drugs.

The chloroethyl group of the biotransformed mustard is very important in the reaction that ensues with macromolecules. Before its reaction, however, the group cyclizes to the imonium (aziridinium) ion, which is the highly reactive alkylating moiety that interacts with the DNA molecule (**Figure 4**). However, it is uncertain whether alkylation by the imonium occurs directly or via rearrangement to the reactive carbonium ion intermediate (Colvin *et al.*, 1999). Since the two chloroethyl groups in the nitrogen mustard drugs are retained in phosphoramide and nornitrogen molecules, a bifunctional reaction with macromolecules ensues. Thus, each drug molecule forms 85.6

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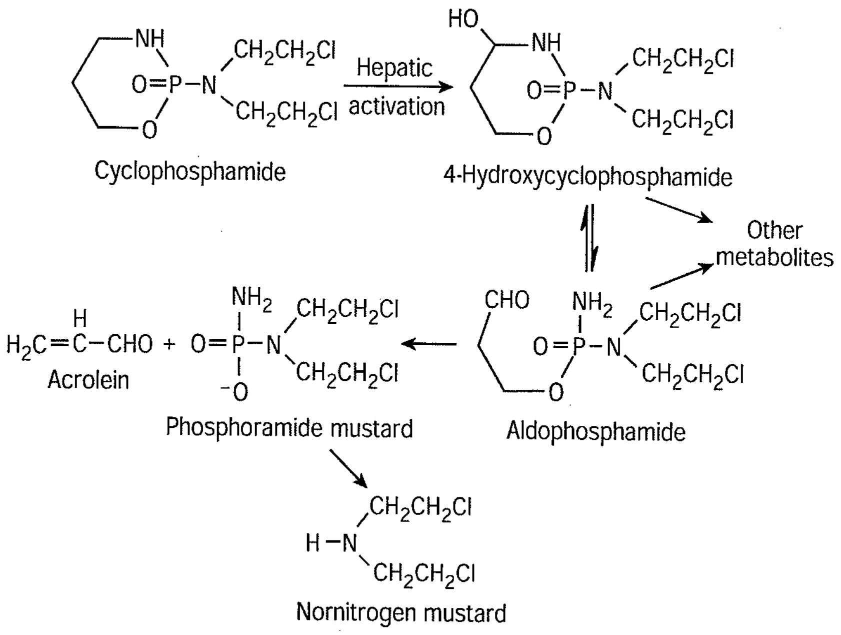
Cyclophosphamide
Hepatic activation
4-Hydroxycyclophosphamide
Other metabolites
Aldophosphamide
Acrolein
Phosphoramide mustard
Nornitrogen mustard

Figure 3 Metabolism of cyclophosphamide to reactive products (adapted from Sladek, 1987 and Pratt *et al.*, 1994).

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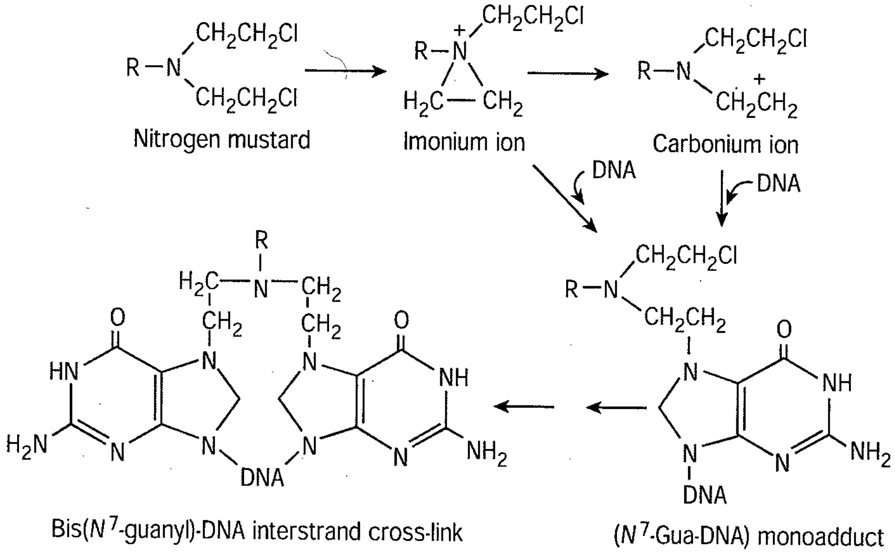

Nitrogen mustard
Imonium ion
Carbonium ion
DNA
DNA
Bis(N^7-guanyl)-DNA interstrand cross-link
(N^7-Gua-DNA) monoadduct

Figure 4 Mechanism of DNA alkylation by a reactive nitrogen mustard molecule and formation of interstrand cross-link (adapted from Sladek, 1987 and Pratt *et al.*, 1994).

adducts with two individual nucleotide bases through a sequential alkylation process; that is, a monofunctional adduct is formed first and this is followed by the second adduct in the opposite strand of the DNA. This bifunctional reaction, thereby, generates an interstrand cross-link between the two strands of DNA in the helix. Both 1,2- and 1,3-cross-links are feasible from an energetic consideration, but it appears that the 1,3-cross-link is favoured by the mustards (Colvin *et al.*, 1999). The interstrand cross-link is considered critical in preventing the two opposing strands from separating during replication, which leads to inhibition of DNA synthesis. Cross-links can also occur on the same strand of DNA and between DNA and protein, but for mustards these lesions are not considered to be cytotoxic (Colvin *et al.*, 1999). Similarly, if the second alkylating reaction is with glutathione, the resulting monofunctional lesion in the DNA also has reduced cytotoxicity. From these considerations, it is reasonable to conclude that the interstrand DNA cross-link is essential for maximal cell killing (Garcia *et al.*, 1988). Indeed, bifunctional interstrand adducts are as much as 100-fold more cytotoxic than monofunctional adducts (Roberts *et al.*, 1968).

85.7 Like cyclophosphamide, the closely related ifosfamide also requires metabolic activation along an identical pathway (Sladek, 1987), but the structural variation in the analogue is such that the rate of hepatic activation is reduced, which, thereby, decreases drug potency. For this reason, about four times as much drug is required to give the same cytotoxic effects as cyclophosphamide (Colvin, 1982). However, the cumulative amount of acrolein produced is substantially greater, which becomes a dose-limiting factor that requires the clinical use of the thiol-containing agent Mesna to inactivate the toxic product. The presence of the electron-withdrawing aromatic ring in melphalan and chlorambucil also reduces the rate of formation of the imonium ion. As a result, the potency of these molecules is also reduced. Metabolic activation appears not to be necessary for these specific nitrogen mustards. However, melphalan is actively transported in certain tumour cells by a high-affinity carrier system that can increase the activity of the molecule (Vistica, 1979). An alternative application of nitrogen mustards in purging leukaemic cells from bone marrow aspirates has required the design of the prodrug 4-hydroperoxycyclophosphamide. This drug does not require metabolic activation for activity, and is, therefore, very effective in an *ex vivo* setting (Yeager *et al.*, 1986).

AZIRIDINES

85.8 The aziridines, also known as ethylenimines, are a family of alkylating agents that contain three-membered aziridine rings. Members of this family include triethylenemelamine, triethylenethiophosphoramide (thio-tepa), and mitomycin C (**Figure 5**). Hexamethylmelamine (Altretamine), which is a close relative of triethylenemelamine, is also a family member, although the classical aziridine ring is absent. The aziridine ring is structurally similar to that present in the reactive imonium ion formed by nitrogen mustards. However, since the aziridine ring does not carry a charge, these drugs are much less reactive than the mustards.

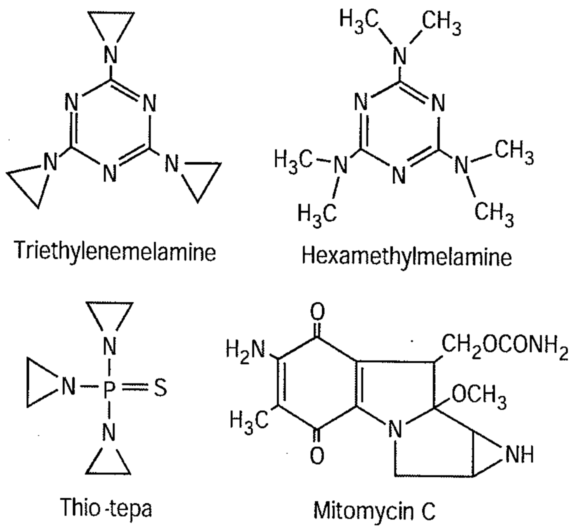


Figure 5 Structures of aziridines and hexamethylmelamine.

I1740

85.9 The aziridines are activated spontaneously or by an enzymatic oxidative reaction. Following activation, alkylation can occur at a number of nucleophilic sites in DNA, RNA, protein and other molecules such as glutathione. With DNA, alkylation reactions of thio-tepa have been reported at a number of sites, including N1 of thymine, O2 of cytosine, N1, N6 and N7 of adenine, and N1, N7 and O6 of guanine (Maanen *et al.*, 2000). However, the preferential target is the N7 position of guanine, with subsequent formation of guanine–guanine (GG) and adenine–guanine (AG) 1,2-interstrand cross-links (Andrievesky *et al.*, 1991). Two possible pathways for the formation of DNA adducts with aziridines are exemplified with thio-tepa (**Figure 6**). One pathway (pathway A in **Figure 6**) involves a sequential reaction that results in cross-link formation. In the second reaction pathway (pathway B), resolved using radiolabelled drug in L1210 leukaemic cells (Egorin and Snyder, 1990; Musser *et al.*, 1992), hydrolytic cleavage liberates the aziridine groups, which induce monofunctional adducts that subsequently lead to DNA strand breaks and cell death. In this respect, thio-tepa functions as a prodrug for the alkylating aziridine molecule (Maanen *et al.*, 2000).

Triethylenemelamine probably undergoes reactions with DNA that are similar to thio-tepa. Mitomycin C, on the other hand, requires an enzymatic reduction to activate the aziridine ring before reaction can occur with DNA initially to form a monofunctional adduct (D'Incalci *et al.*, 1992; Pratt *et al.*, 1994). The preferential alkylation site for this initial reaction appears to be the N2 position of guanine. A second alkylation reaction with the opposite DNA strand follows the spontaneous intramolecular elimination of the carbamate group and results in interstrand cross-links between guanine bases (**Figure 7**). However, alkylation is preferred in 5′C–G3′ sequences to give the 1,2-GG cross-links in DNA. Metabolic activation also plays an important role in activating hexamethylmelamine. Hepatic mixed function oxidases sequentially metabolize the methyl groups in the molecule to alcohol derivatives, which rearrange to reactive iminium ions that then alkylate guanine bases. In **Figure 8**, the 85.10

I741

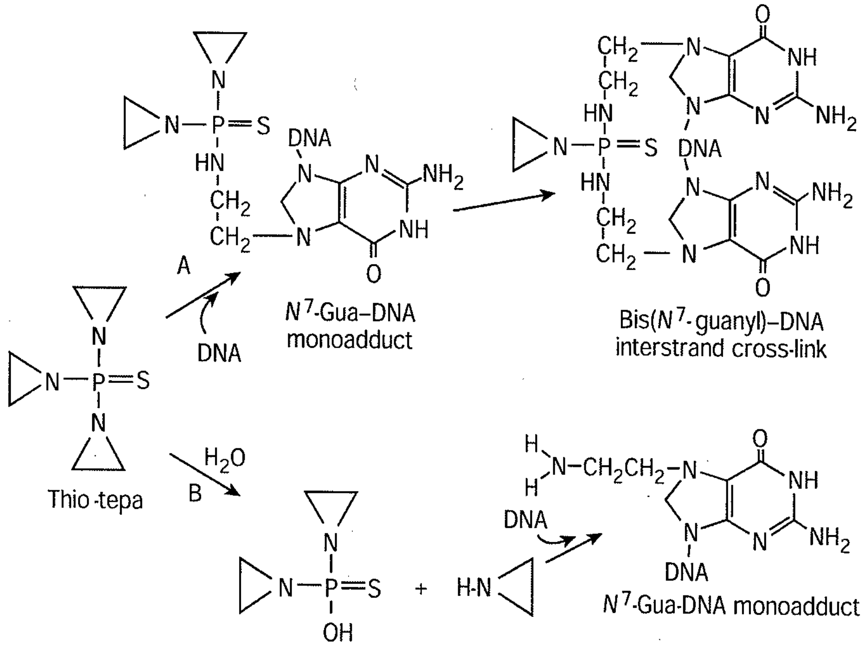

A
B
DNA
H_2O
Thio-tepa
N^7-Gua–DNA monoadduct
Bis(N^7-guanyl)–DNA interstrand cross-link
N^7-Gua-DNA monoadduct

Figure 6 Mechanisms involved in the formation of DNA interstrand cross-links (pathway A) and monofunctional adducts (pathway B) by thio-tepa (adapted from Maanen *et al.*, 2000).

I742

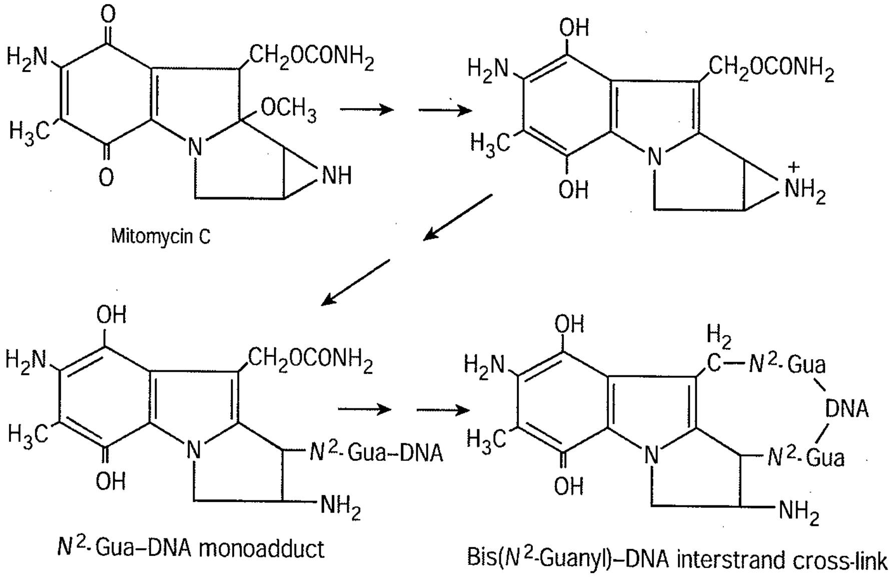
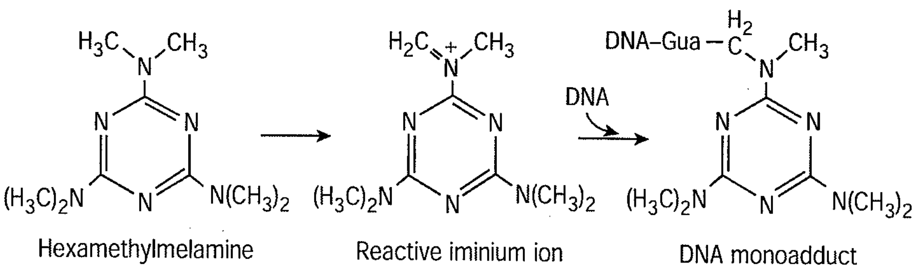

Mitomycin C
N^2-Gua–DNA monoadduct
Bis(N^2-Guanyl)–DNA interstrand cross-link

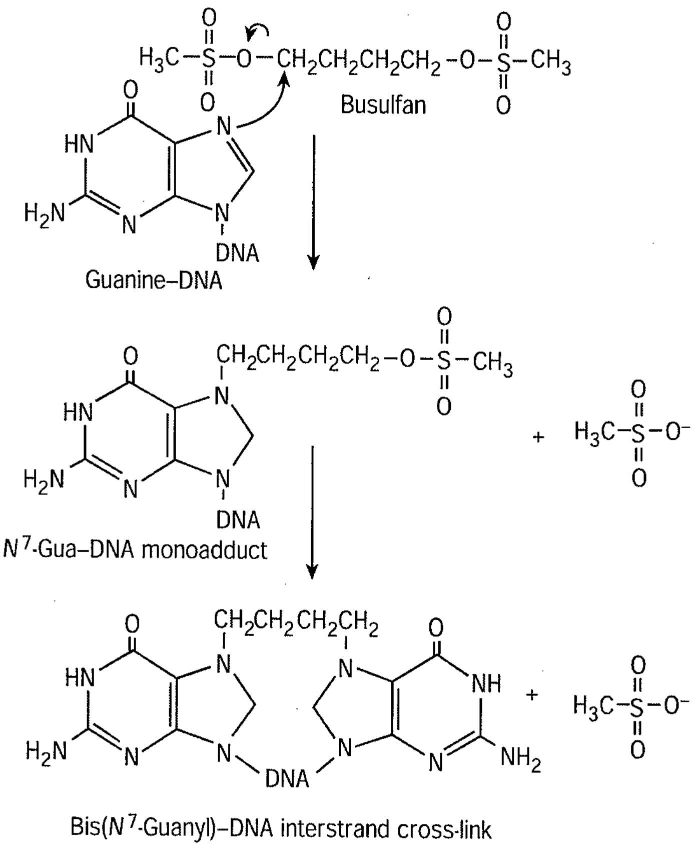
Figure 7 Reaction of mitomycin C with DNA to form cross-links between guanine bases (adapted from Pratt *et al.*, 1994).

conversion of hexamethylmelamine to pentamethylmelamine is demonstrated, but subsequent metabolism of this product can lead to the loss of all six methyl groups and potential generation of an iminium ion from each methyl group metabolized. Hexamethylmelamine derivatives, such as trimelamol, have also been designed that do not appear to require metabolic activation for generation of the reactive iminium ion (Siddik and Newman, 1994).


DNA
Hexamethylmelamine
Reactive iminium ion
DNA monoadduct

I743

Figure 8 Conversion of HMM to an iminium ion and the formation of DNA adduct (adapted from Pratt *et al.*, 1994).


Busulfan
Guanine–DNA
N^7-Gua–DNA monoadduct
Bis(N^7-Guanyl)–DNA interstrand cross-link

I744

Figure 9 Mechanism of formation of DNA interstrand cross-links induced by busulfan.

ALKYL SULFONATES

85.11 Busulfan is the best known of the alkyl sulfonates, and has a linear symmetrical chemical structure that facilitates cross-link formation. However, the mechanism of alkylation of this molecule is different. Unlike the mustards and the aziridines, which must first generate reactive species, busulfan interacts directly with the N7 position of guanine and leads to the formation of DNA mono- and then bi-adducts, with release of methyl sulfonate groups (**Figure 9**). Interstrand cross-links between guanines have been demonstrated for busulfan (Tong and Ludlum, 1980) and, as with the mustards, this is considered the cytotoxic lesion (Bedford and Fox, 1983).

NITROSOUREAS

Much of the early focus on nitrosoureas as antitumour agents came from studies of Montgomery and co-workers at the Southern Research Institute in Birmingham, 85.12

AL, USA (Reed, 1987). The extensive structure–activity studies over many years established the foundation that eventually led to the discovery of the more useful 2-chloroethylnitrosoureas (CENUs) that are currently in clinical use. A number of nitrosoureas are of clinical interest, and include BCNU (carmustine), CCNU (lomustine), methyl-CCNU (semustine) and chlorozotocin (**Figure 10**).

85.13 In general, the CENUs are highly unstable and rapidly undergo spontaneous transformation to yield a number of products (**Figure 11**). A most significant product, however, is the highly unstable 2-chloroethyldiazene hydroxide, which transforms to the alkylating 2-chloroethylcarbonium ion (Ludlum, 1997). Although this reactive ion can alkylate nucleophilic sites in DNA to yield a number of modified DNA bases, the N7 position of guanine appears to be a predominant site for alkylation, particularly when this base is in the middle of a run of three or more guanines in DNA (Reed, 1987; Lemoine *et al.*, 1991). In contrast to other DNA-reactive agents, the CENUs also alkylate the O6 site of guanine to a large extent. The significance of O6 alkylation can be recognized from the knowledge that cytotoxicity correlates inversely with cellular activity of the DNA repair enzyme O^6-alkylguanine–DNA alkyltransferase, which removes the monofunctional O6 adduct from the DNA (Pratt *et al.*, 1994). Thus, when the O^6-alkyltransferase is overexpressed, sensitivity of tumour cells to CENUs diminishes. The DNA monoadducts are chloroethyl derivatives, but substantial amounts of hydroxyethyl adducts are also formed (**Figure 11**). It is possible that hydroxyethyl adducts could arise from hydrolysis of chloroethylated bases, but it appears more likely that other reactive intermediates of CENUs are involved in the transfer of

1745

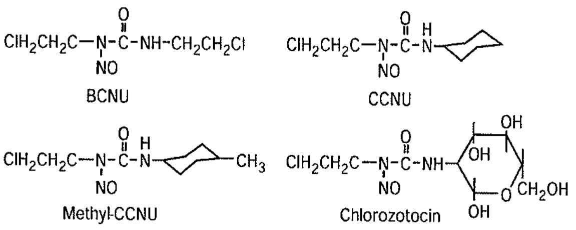

BCNU
CCNU
Methyl-CCNU
Chlorozotocin

Figure 10 Structures of selected 2-choloroethylnitrosourea antitumour agents.

1746

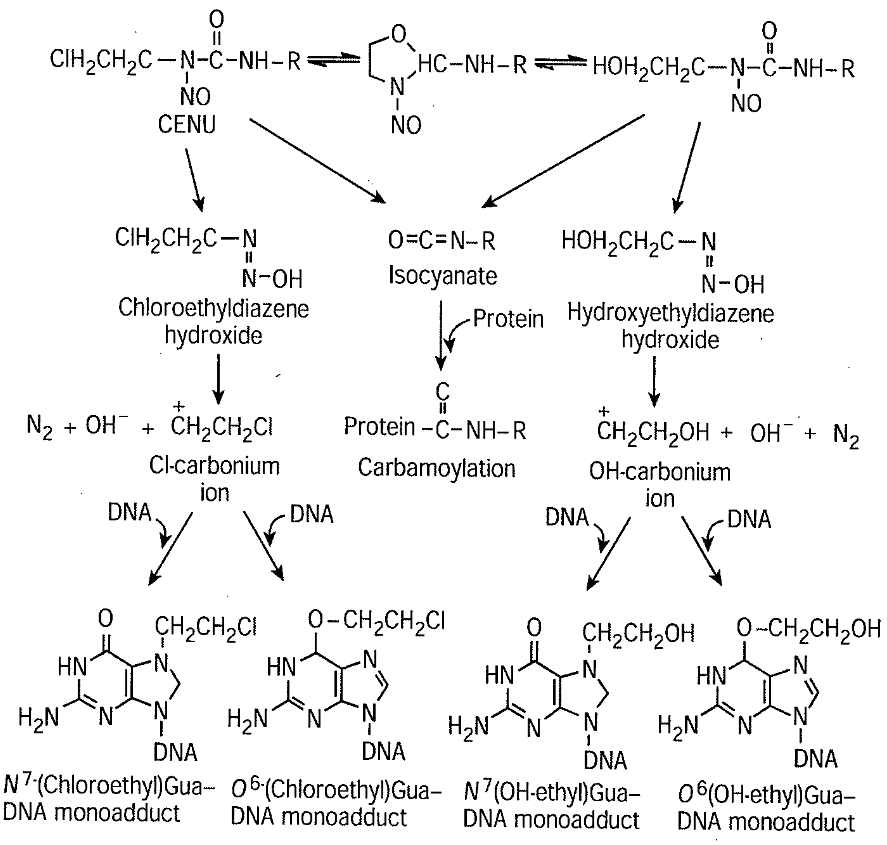

CENU
Chloroethyldiazene hydroxide
Isocyanate
Protein
Hydroxyethyldiazene hydroxide
Cl-carbonium ion
Carbamoylation
OH-carbonium ion
DNA
DNA
DNA
DNA
N7-(Chloroethyl)Gua-DNA monoadduct
O6-(Chloroethyl)Gua-DNA monoadduct
N7(OH-ethyl)Gua-DNA monoadduct
O6(OH-ethyl)Gua-DNA monoadduct

Figure 11 General reaction of a 2-chloroethylnitrosourea (CENU) with DNA to form monoadducts as the initial step in cross-link formation (adapted from Eisenbrand *et al.*, 1986, Pratt *et al.*, 1994 and Ludlum, 1997).

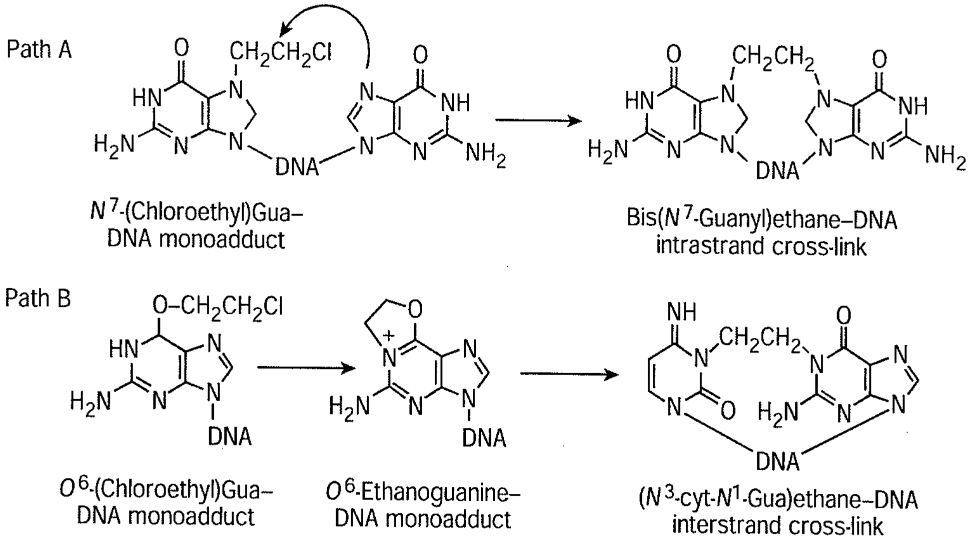

Path A
N^7-(Chloroethyl)Gua–DNA monoadduct
Bis(N^7-Guanyl)ethane–DNA intrastrand cross-link
Path B
O^6-(Chloroethyl)Gua–DNA monoadduct
O^6-Ethanoguanine–DNA monoadduct
(N^3-cyt-N^1-Gua)ethane–DNA interstrand cross-link

Figure 12 Mechanisms responsible for the conversion of monofunctional adducts of CENU to GG intrastrand (pathway A) and CG interstrand (pathway B) cross-links (adapted from Ludlum, 1997).

hydroxyethyl groups to DNA. One likely explanation is that the CENUs can cyclize (**Figure 11**), and during decyclization the chloride group is replaced by the hydroxyl group, which can then form the alkylating 2-hydroxyethyl carbonium ion (Eisenbrand *et al.*, 1986).

85.14 The initial monofunctional adduct formed by the CENU is converted to an alkyltransferase-resistant 1,2-cross-link through labilization of the alkylating chloroethyl group on the initial site and reaction with a nucleophilic site on a second DNA base (**Figure 12**, pathway A). This explains why CENUs, such as CCNU, with only a single chloroethyl side chain have the capacity to cross-link DNA. Reaction kinetics indicate that the initial alkylation to form the DNA monoadduct occurs very rapidly (usually within minutes), whereas the conversion to the cross-link can take 6–12 h. Chemical structures of two DNA lesions have been identified as guanine–guanine (through N7 positions) and N^3-cytosine-N^1-guanine (CG) 1,2-cross-links. Although the chemical reaction leading to the bis-guanine (GG) cross-link at the N7 positions is consistent with the characterized N^7-guanine monofunctional adduct, the CG cross-link through the N1 position of guanine is not as straightforward to comprehend, particularly since alkylation at the N1 site is rare. It is most likely that the CG crosslink occurs through an initial O^6-guanine adduct, which cyclizes to the O^6-ethanoguanine intermediate that then reacts with cytosine (**Figure 12**, pathway B). Steric considerations suggest that the CG cross-link is interstrand and the GG cross-link is intrastrand (Ludlum, 1997). Irrespective of their nature, both GG and CG cross-links correlate strongly with cytotoxicity.

85.15 A second interesting product of spontaneous CENU transformation is the isocyanate species (**Figure 11**), which is formed in varying amounts depending on the chemical structure of the CENU. Although isocyanates can carbamoylate a range of proteins at the ε-amino group of lysine, including nuclear histone and nonhistone proteins, there is no correlation between carbamoylation activity and cytotoxicity (Lemoine *et al.*, 1991). Chlorozotocin, for instance, has low carbamoylating activity, but retains antitumour activity. However, there is disagreement whether carbamoylation reaction contributes to the side effects of CENUs. Although the rate and extent of carbamoylation of protein may not be associated with bone marrow toxicity of some nitrosoureas (Reed, 1987), this does not necessarily preclude other CENUs that possess a distinctly different carbamoylating isocyanate function in the molecule from inducing myelotoxicity (Ali-Osman *et al.*, 1985).

PLATINUM-BASED AGENTS

The platinum drug cisplatin (*cis*-diamminedichloroplatinum(II)) is perhaps one of the most effective antitumour agents currently in clinical use. Although the drug had previously been known as Peyrone's salt for over 100 years, it was not until 1969 that its antitumour effects were first recognized through a serendipitous finding. In an experiment designed to determine how *E. coli* would behave in an electric field, Rosenberg and colleagues (Rosenberg, 1980) passed an electrical current via platinum electrodes through a bacterial culture, which contained nutrients that included ammonium chloride as a source of nitrogen. It was noted that the bacteria stopped dividing, but continued to grow and became filamentous. Subsequent investigations to explain this observation led 85.16

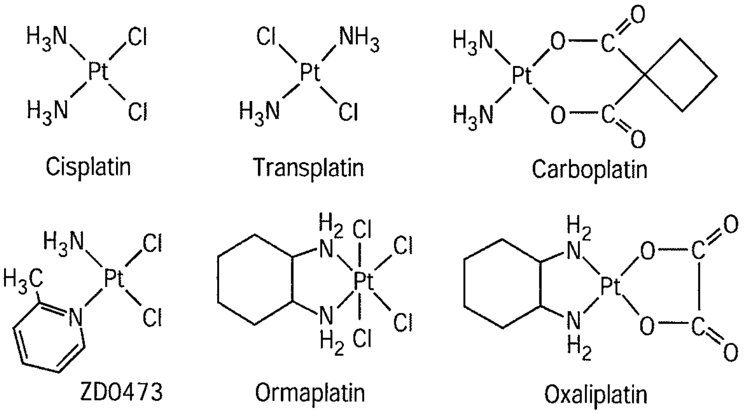


Figure 13 Structures of cisplatin and selected analogues of clinical and mechanistic interests.

to the isolation from the culture of several divalent and tetravalent platinum-containing products that formed from the reaction between the electrodes and the culture medium (presumably ammonium chloride). The most effective of the agents was identified as cisplatin, which was subsequently developed as an antitumour agent. The drug became approved for clinical trials in 1972.

85.17 There is no question that cisplatin has had a major impact in the treatment of several important cancers, such as those of the ovary, testes and head and neck (Prestayko *et al.*, 1979), but its clinical utility can often be compromised by side effects and the onset of tumour drug resistance. It was indeed the dose-limiting nephrotoxicity that led to the search for a less toxic platinum analogue. The eventual identification of the clinically active carboplatin in the early 1980s by Harrap and his colleagues was a result of an intensive laboratory-based effort that required an initial examination of over 300 analogues (Kelland *et al.*, 1999). Although carboplatin has been important in overcoming the irreversible renal damage and the peripheral neuropathy associated with cisplatin use in patients, it is, however, fully cross-resistant with the parent molecule (Gore *et al.*, 1989; Eisenhauer *et al.*, 1990). Therefore, greater attention has been devoted recently to identify analogues capable of circumventing cisplatin resistance, and a few have been introduced into clinical trials with various degrees of success (Kelland *et al.*, 1999). The 1,2-diaminocyclohexane (DACH)-based oxaliplatin is fulfilling its potential against specific refractory cancers (Faivre *et al.*, 1999), but the underlying basis for its activity is yet to be defined. Indeed, this and other analogues, such as ZD0473 (Kelland *et al.*, 1999), are still under active clinical investigations and, regardless of the fact that current investigations are intense, it may be some time before their mechanism of action will become fully appreciated. However, it is useful to note that according to the results of the DISCOVERY computer program analysis by the National Cancer Institute in the USA, cisplatin and its analogues fall into at least 13 clustered regions, each reflecting a distinct mechanism of action (Tanimura *et al.*, 1995). These mechanisms are also not known at the present time. Indeed, almost 30 years after its clinical acceptance as a potent antitumour drug, we are still searching for answers to explain exactly how cisplatin works. Therefore, this section will focus primarily on our present understanding of the mechanism of action of cisplatin.

Some of the platinum drugs of interest, either clinically or from the perspective of understanding the mechanism of action, are shown in **Figure 13**. Cisplatin is a square-planar inorganic molecule, which has the central platinum in a divalent state. Other platinum(II) agents have similar configurations. In contrast, the tetravalent platinum(IV) compounds, such as ormaplatin (tetraplatin), have an octahedral structure, but they are considered as prodrugs for the corresponding active platinum(II) structures. Cisplatin has a rigid structure, with two labile chloro and two stable ammine ligands in a *cis* configuration. This is critical for antitumour activity, as the isomer transplatin, with a *trans* geometry, is relatively ineffective. A few active experimental *trans*-platinum(II) agents, however, transcend the absolute requirement for a *cis* configuration (Perez *et al.*, 1999), but the reason for this is unclear. The cytotoxic activity of cisplatin has sparked considerable interest in other metal-based agents, but none of the possible metal alternatives, including gold, ruthenium, rhodium and palladium, provide the optimal chemical environment for active antitumour drugs. 85.18

Chemistry of Cisplatin as a Basis for Activity

Cisplatin is considered a very potent antitumour agent, yet from a chemical perspective the molecule itself is inert, and unable to react with biological macromolecules. Like some alkylating agents, the neutral drug molecule needs to be converted to a reactive form. This occurs non-enzymatically in solution, where displacement reactions 85.19

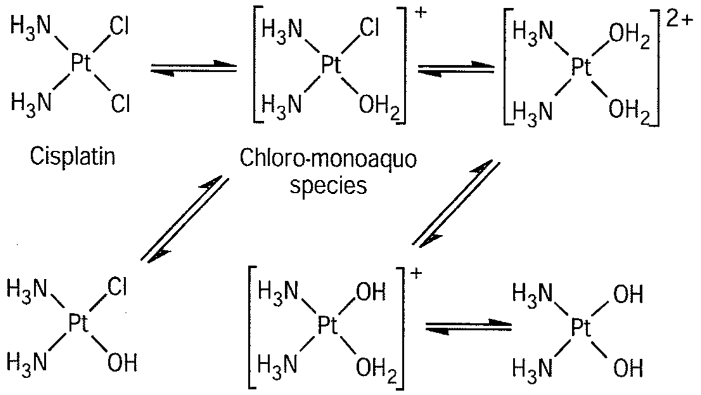


Figure 14 Conversion of cisplatin to positively charged reactive species via reversible aquation reactions.

result in stepwise exchange of the labile chloro ligands with water molecules (el Khateeb *et al.*, 1999; Kelland, 2000). Such aquations also occur with other platinum analogues, and lead to several species that exist in equilibrium, as exemplified with cisplatin in **Figure 14**. The charged aquated species are highly reactive, but the chloromonoaquo species is the most significant from the perspective of interaction with DNA at physiological pH. The reactive aquated species, however, can also be inactivated through nonspecific interaction with many endogenous nucleophilic molecules and macromolecules, such as glutathione, methionine, metallothionein and protein. In the case of carboplatin, which has a more stable bidentate cyclobutanedicarboxylate ligand, the aquation reaction is much slower. This reduces drug potency, which thereby requires a greater dose for an equivalent antitumour effect. Indeed, since the final reactive species arising from cisplatin and carboplatin are identical, the slower rate of aquation may be the underlying basis for the reduced renal toxicity and peripheral neuropathy of carboplatin. This has led to the concept that high peak plasma concentrations of cisplatin are cytotoxic to both normal and tumour cells, whereas low sustained levels are equally effective against tumour cells but less toxic to normal cells. Support for the concept has come from clinical studies, which demonstrate the potential for an increase in the therapeutic index when cisplatin is given as a slow continuous infusion over several days (Salem *et al.*, 1978, 1984).

85.20 Although activation is essential for activity, a substantially rapid generation of the reactive species is in general not conducive to antitumour effects. This may be particularly relevant in understanding why the highly reactive gold- or palladium-based agents are ineffective as antitumour agents. It is likely that these compounds are rapidly inactivated during nonspecific random interactions with plasma proteins and other components, and are therefore unable to reach the tumour site in sufficient concentrations to have any effect. Similarly, the clinical failure of the platinum(IV) agent ormaplatin could be ascribed to its rapid reduction to the platinum(II) form in the plasma and immediate inactivation of the transformed species through irreversible, noncytotoxic interactions with macromolecules such as plasma proteins (Siddik *et al.*, 1999).

DNA as a Target of Platinum Drugs

Studies conducted by several investigators, including 85.21 Roberts and Pera (1983), leave little doubt that DNA is the primary target of cisplatin and other platinum agents. However, very little is known regarding the chemical form of cisplatin that reaches the nucleus. It is likely that the neutral uncharged species is the form that traverses the nuclear membrane. Although cisplatin enters cells through a predominantly nonsaturable passive diffusion process (Kelland, 2000), it is not known if a similar process also operates in nuclear drug uptake. Once inside the nucleus, the activated form of cisplatin interacts sequentially with nucleophilic sites on purine bases in DNA. First, as soon as the monoaquated species of cisplatin is formed, it reacts immediately with a DNA base (preferentially N7 of guanine) to form a monofunctional adduct. Such platinated adducts are considered inactive, as ascertained, for instance, from the inability of monofunctional adducts of cisplatin or transplatin to terminate RNA synthesis by bacterial RNA polymerases on DNA templates (Lemaire *et al.*, 1991; Brabec and Leng, 1993). The remaining chloride ligand linked to platinum in the monoadduct is then hydrolysed, and the resulting aquated species interacts with a second nucleophilic site to form DNA and DNA–protein cross-links (**Figure 15**). Both 1,2- and 1,3-intrastrand DNA cross-links have been observed. The

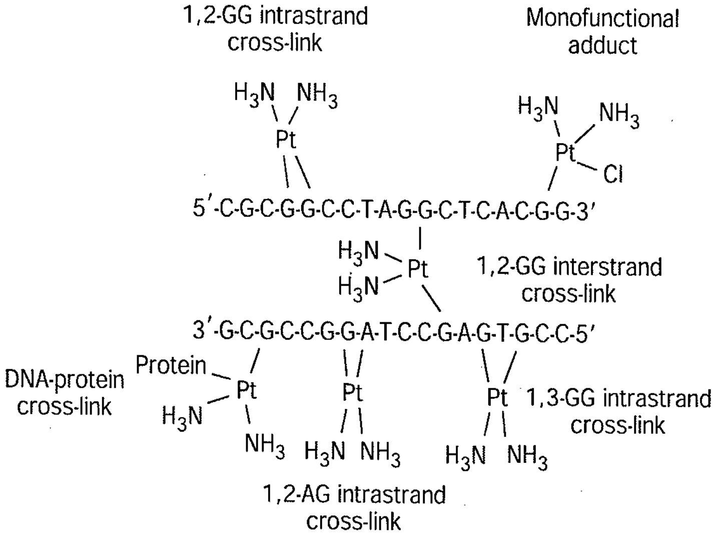

1,2-GG intrastrand cross-link
Monofunctional adduct
H_3N NH_3
Pt
H_3N NH_3
Pt
Cl
5′-C-G-C-G-G-C-C-T-A-G-G-C-T-C-A-C-G-G-3′
H_3N
Pt
H_3N
1,2-GG interstrand cross-link
3′-G-C-G-C-C-G-G-A-T-C-C-G-A-G-T-G-C-C-5′
DNA-protein cross-link
Protein
Pt
H_3N
NH_3
Pt
H_3N NH_3
Pt
H_3N NH_3
1,3-GG intrastrand cross-link
1,2-AG intrastrand cross-link

Figure 15 Types of DNA adducts and cross-links induced by cisplatin.

1,2-interstrand DNA cross-links between opposite guanine bases are formed preferentially in 5′G–C3′ (G–C) sequences of both strands of linear DNA, but not in 5′C–G3′ (C–G) sequences as preferred by mitomycin C. The preference for G–C sequence for the formation of interstrand platinum cross-link is probably due to the relatively shorter distance between opposite guanines in G–C sequences (Malinge *et al.*, 1999). Interestingly, interstrand cross-links are formed in both G–C and C–G sequences in supercoiled DNA, and this suggests that DNA topology can regulate interstrand platination reaction.

85.22 There is still uncertainty whether interstrand or intrastrand DNA cross-links are the cytotoxic lesions. Although interstrand cross-links can lead to biological effects, such as inhibition of transcriptional activity of prokaryotic and eukaryotic RNA polymerases on a damaged DNA template (Corda *et al.*, 1991), the bulk of the evidence suggests that intrastrand adducts provide the strongest basis for the cytotoxic action of cisplatin, This is consistent with the knowledge that the relatively inactive transplatin cannot form intrastrand cross-links (Roberts and Friedlos, 1987). The substantial interest in intrastrand cross-links is also a result of biochemical analysis, which demonstrate that 1,2-intrastrand AG and GG cross-links account for about 85–90% of all DNA adducts (Kelland, 1993). In contrast, the 1,3-intrastrand GXG cross-links (where X is any nucleotide), interstrand GG cross-links and monofunctional adducts each make up about 2–6% of the platinum bound to DNA. The level of the AXG intrastrand adduct, on the other hand, is negligible. Although interstrand adduct levels are relatively low, they have also been correlated directly to cytotoxicity and, therefore, cannot be totally discounted (Roberts and Friedlos, 1987). The interstrand cross-links, on the other hand, are relatively unstable and convert to the more stable intrastrand form, with a half-life of about 29 h (Perez *et al.*, 1997; Malinge *et al.*, 1999). Similar levels of monoadducts and interstrand and intrastrand bi-adducts are also found for the analogue DACH-sulfatoplatinum(II) in an *in vitro* system (Jennerwein *et al.*, 1989). Since cells resistant to cisplatin have only a low level of cross-resistance to this and other similar DACH-containing analogues (Eastman, 1987), it is reasonable to conclude that if the mechanism of action is at the DNA level, then the chemically specific adducts of cisplatin and DACH-based platinum agents (e.g. oxaliplatin) (**Figure 16**) must be a major determinant of the differential mode of action between the platinum drugs. Similarly, the chemical nature of adducts formed by cisplatin and carboplatin are identical, which is consistent with the knowledge that cisplatin-resistant tumours are cross-resistant to carboplatin. Compared with interstrand and intrastrand DNA cross-links, DNA–protein cross-links have been dismissed from playing a role in the cytotoxic process, partly on the basis of the finding that such lesions are formed extensively by the inactive agent transplatin (Zwelling *et al.*, 1979).

Effect of Cross-links on DNA Structure and Damage Recognition

85.23 It is widely understood that cross-linked adducts induced by cisplatin disrupt replication and transcriptional processes. Even just a few cross-links in the entire genome can be sufficient to inhibit DNA replication (Heiger-Bernays *et al.*, 1990). Such biological effects, however, do not necessarily correlate directly with cytotoxic effects. Therefore, formation of cross-link lesions should merely

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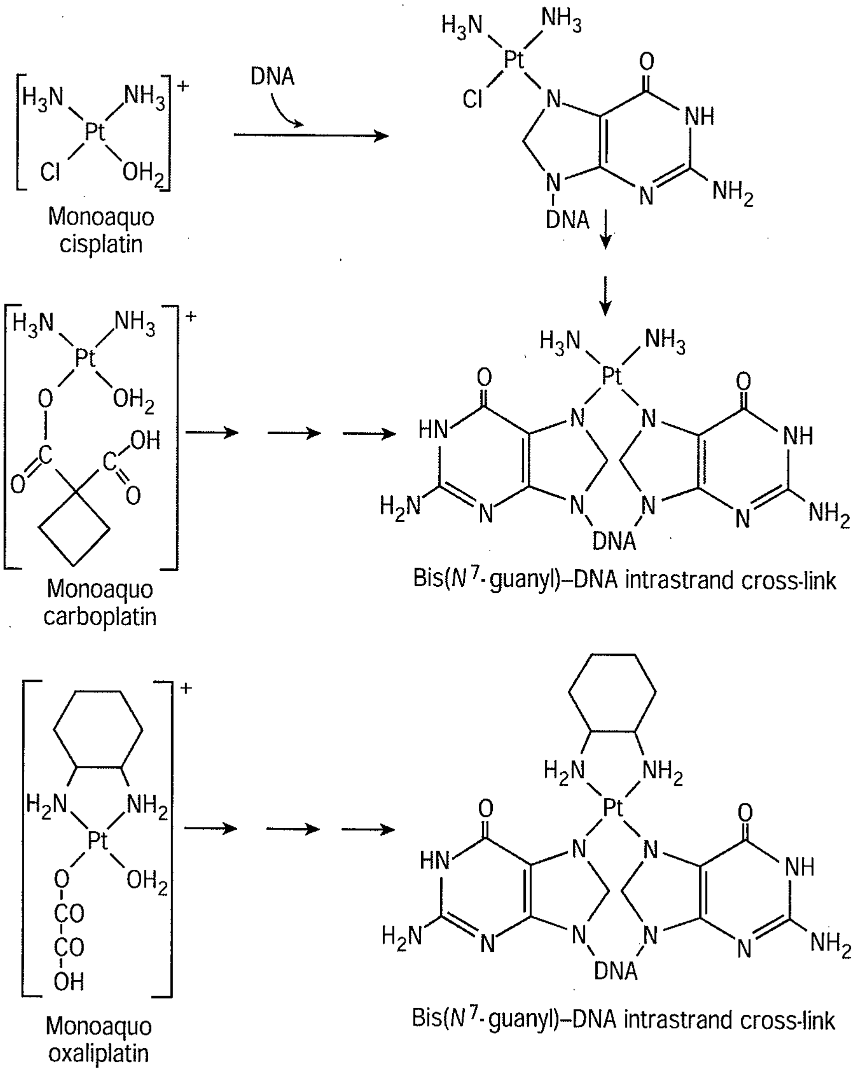
DNA
Monoaquo cisplatin
DNA
Monoaquo carboplatin
Bis(N7-guanyl)–DNA intrastrand cross-link
Monoaquo oxaliplatin
Bis(N7-guanyl)–DNA intrastrand cross-link

Figure 16 Cross-links between guanine bases induced by cisplatin, carboplatin and oxaliplatin. Note that cisplatin and carboplatin form an identical cross-link, whereas the cross-link of oxaliplatin is structurally very different by virtue of the bulky 1,2-diaminocyclohexane (DACH) group in the adduct.

be considered as the initial step in the complex process leading to cell death. Both interstrand and intrastrand cross-links induce local unwinding and bending in the DNA double helix. The AG, GG and GXG intrastrand bi-adducts of cisplatin unwind DNA by 13–23° and bend the double helix by 32–34° (Bellon *et al.*, 1991). Interstrand cross-links, on the other hand, induce much greater effects: unwinding of 79° and greater, and bends of 45–47° have been reported (Malinge *et al.*, 1999). Such physicochemical characteristics may determine which signal transduction pathways are activated by interstrand and intrastrand adducts to induce cytotoxicity. Activation of these pathways probably occurs through special proteins with damage recognition properties that recognize the distinct distortions in the DNA and thereby affect cellular events, such as cell cycle arrest and apoptosis (a programmed form of cell death).

More than 20 different damage recognition proteins 85.24 have been identified, and some specificity has been demonstrated by these proteins for DNA adducts of cisplatin and analogues. The mismatch repair (MMR) complex proteins, for instance, bind to cisplatin-induced DNA cross-links with much greater affinity than to those formed by oxaliplatin. The MMR appears to be essential for cisplatin sensitivity but is not involved in the mechanism of oxaliplatin-induced cytotoxicity (Chaney and Vaisman, 1999). Another important protein involved in recognition is the high mobility group 1 (HMG1) protein that recognizes cross-links of both cisplatin and oxaliplatin (Donahue *et al.*, 1990), but the relative affinity again appears to be greater for those of cisplatin (Chaney and Vaisman, 1999). HMG1 binds to both intrastrand AG and GG adducts, but not to intrastrand GXG or monofunctional adducts. Interestingly, HMG1 also recognizes interstrand cross-links induced by cisplatin, but fails to interact with 1,1-cross-link of transplatin formed between guanine and the complementary cytosine residue (Kasparkova and Brabec, 1995). The TATA-binding protein (TBP), on the other hand, binds to adducts of both cisplatin and oxaliplatin with similar affinity (Chaney and Vaisman, 1999). In contrast, other damage recognition proteins, such as the Ku subunit of DNA-dependent protein kinase (DNA-PK), bind to DNA damage induced by either the active cisplatin or the inactive transplatin (Turchi *et al.*, 1999). It is highly likely that each recognition protein initiates a specific molecular event, which may lead to cell death. Thus, differences between platinum analogues in their mode of action may be a result of differential recognition of individual distortions in DNA caused by drug-distinct bending and/or unwinding at the site of platination by the platinum analogue. The process, however, is probably more complex. For instance, intrastrand GG and GXG adducts induce similar bending and unwinding in DNA, but are differentially recognized by HMG1. It is very likely, therefore, that other factors contribute to the recognition process.

The Role of the Tumour Suppressor p53

85.25 How the damage recognition proteins determine the fate of cells is not entirely clear. They have been implicated in shielding DNA adducts from repair that has the effect of increasing persistence of damage to facilitate cytotoxicity. This is consistent with the inverse relationship that an increase in nucleotide excision repair capacity of cells leads to a decrease in sensitivity of tumour cells to cisplatin. On the other hand, damage recognition proteins may play a role in activating signalling pathways, which affect a number of molecular events, including regulation of the tumour suppressor p53 protein (Kastan *et al.*, 1991; Hainaut, 1995; Jayaraman *et al.*, 1998). Normally, p53 is maintained intracellularly at very low levels or in an inactive state by its binding to the Mdm2 protein (Lakin and Jackson, 1999). When DNA is damaged by cisplatin, binding between Mdm2 and p53 is disrupted by phosphorylation of the tumour suppressor and results in p53 induction by virtue of a greater metabolic stability of the free p53 than of the p53–Mdm2 complex (Fritsche *et al.*, 1993; Shieh *et al.*, 1997; Lakin and Jackson, 1999). Once induced by the DNA damaging agents, p53 can transcriptionally activate DNA in a sequence-specific manner, eventually to give rise to other regulatory proteins such as $p21^{Waf1/Cip1}$ or Bax that can facilitate cell cycle arrest or cell death, respectively (Sionov and Haupt, 1999). However, the transcriptional activation by p53 is carefully orchestrated to provide a sequence of events that first results in cell cycle arrest through activation of cell cycle checkpoints to prevent not only DNA synthesis on a drug-damaged DNA template, but also segregation of damaged chromosomes during mitosis. If the cell cannot repair the damaged DNA, then apoptotic events are activated.

That p53 is a critical protein in protecting the genome 85.26 and in preventing mutations in DNA from being passed on to daughter cells, comes from the realization that about 50% of all cancers have mutated p53, which has lost normal regulatory functions (Hollstein *et al.*, 1991; Kastan *et al.*, 1991; Hartwell and Kastan, 1994; Oltvai and Korsmeyer, 1994). In cancer chemotherapy, the intrinsic function of p53 to induce cell death and prevent damaged DNA to be propagated to normal daughter cells is exploited. It is not surprising, therefore, that the presence in tumours of mutant p53, compared with wild-type p53, reduces survival rates in patients treated with cisplatin for stage III/IV ovarian cancers (van der Zee *et al.*, 1995). In such cases, combining cisplatin with gene therapy to restore wild-type p53 has become a viable therapeutic option. Presence of wild-type p53 in tumours, however, does not necessarily ensure greater sensitivity to cisplatin. Indeed, some tumour cell lines bearing wild-type p53 are highly resistant to cisplatin, and this has been attributed to a defective signalling pathway that fails to activate p53 following DNA damage as a result of cross-link formation. Interestingly, a DACH-containing analogue, (1*R*,2*R*)-DACH-(*trans*-diacetato)(dichloro)platinum(IV), is able to activate the dormant p53 and induce cytotoxicity (Hagopian *et al.*, 1999; Siddik *et al.*, 1999), which consolidates the belief that signalling transduced by DNA damage are different for cisplatin and such mechanistically distinct analogues. Although the activity of this DACH-based platinum(IV) compound was dependent on wild-type p53, it is known that cell cycle arrest and cell death can also occur in a p53-independent manner, which is not well understood (Michieli *et al.*, 1994; Zhang *et al.*, 1995; Segal-Bendirdjian *et al.*, 1998; Haapajarvi *et al.*, 1999). Furthermore, under certain conditions, inactivation of p53 can enhance cytotoxic sensitivity to cisplatin (Fan *et al.*, 1995; Hawkins *et al.*, 1996). These findings add credence to the understanding that cisplatin-induced cell death is a very complex process that will require greater knowledge to unravel the interplay between several signalling pathways that eventually determine whether a cell lives or dies.

Induction of Apoptosis

Members of the Bcl-2 family are also involved in the 85.27 mechanism of action of cisplatin. Specific members are localized in the mitochondria and have either proapoptotic (Bax, Bak, Bid, Bim) or antiapoptotic (Bcl-2, Bcl-XL, Bcl-W) functions (Farrow and Brown, 1996; Hanahan and Weinberg, 2000). These proteins form either homodimers

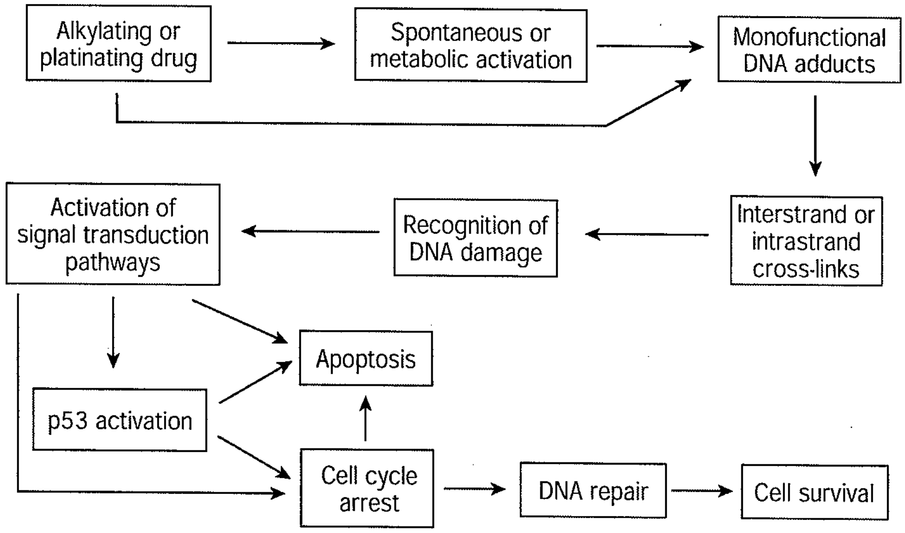
Alkylating or platinating drug
Spontaneous or metabolic activation
Monofunctional DNA adducts
Interstrand or intrastrand cross-links
Recognition of DNA damage
Activation of signal transduction pathways
Apoptosis
p53 activation
Cell cycle arrest
DNA repair
Cell survival

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Figure 17 A general scheme for DNA-interactive agents that proposes critical events leading to DNA damage and subsequent cell survival or apoptotic form of cell death.

(such as Bcl-2/Bcl-2) or heterodimers (e.g. Bcl-2/Bax) depending on the levels present of each component. Only an excess level of homodimers can either inhibit (e.g. Bcl-2/Bcl-2) or induce (e.g. Bax/Bax) apoptosis. Although there is no information available to indicate whether cisplatin can directly modulate levels of the antiapoptotic protein, there is evidence of a significant drug-mediated effect on Bax levels through transactivation of the *bax* gene by wild-type p53. Thus, an increase in the Bax to Bcl-2 ratio by cisplatin-induced p53 has been reported to activate the apoptotic process (Eliopoulos *et al.*, 1995). However, caution needs to be exercised in extrapolating experimental results to the clinic. For instance, the demonstration that experimental overexpression of *bcl-2* in tumours leads to the expected cisplatin resistance (Strasser *et al.*, 1994; Herod *et al.*, 1996; Miyake *et al.*, 1999) is in sharp contrast to a clinical study, which reported that cisplatin surprisingly improved survival of patients with ovarian cancer that demonstrated increased *bcl-2* gene expression (Herod *et al.*, 1996). Our present understanding indicates that proapoptotic homodimers affect cisplatin-induced apoptosis by first stimulating the mitochondria to release cytochrome *c*, which in turn activates a series of proteases that includes caspase-1, -3 and -9 (Kondo *et al.*, 1995; Henkels and Turchi, 1999; Gebauer *et al.*, 2000; Hanahan and Weinberg, 2000). These proteases appear to be the final effectors of drug-mediated apoptotic cell death.

CONCLUSION

85.28 From the above discussions, we can formulate a general understanding for the mechanism of action of alkylating and platinating agents. Although much of the information has been derived from studies with platinum-based drugs, the general principles most likely apply to alkylating agents also. Once these antitumour agents are activated, they damage DNA by forming monofunctional adducts and interstrand and intrastrand cross-links, which cause DNA to unwind and/or bend. Such distortions are then recognized by specialized DNA damage recognition proteins, and a cascade of events is activated that leads to p53-dependent or -independent cell cycle arrest to allow time for DNA repair. If repair is incomplete, p53-dependent or -independent programmed cell death (apoptosis) is initiated to complete an orderly process of cell destruction. **Figure 17** summarizes this general sequence of events, and suggests that any factor interfering with this scheme, such as reduced adduct formation (e.g. drug inactivation by glutathione) or persistence (e.g. enhanced repair), reduced recognition of damage (e.g. mutation in mismatch repair complex), aberrant signal transduction pathways (e.g. mutation in *p53*), and reduced apoptotic activity (e.g. *p53* mutation or increased *bcl-2* overexpression), will lead to resistance to alkylating and platinating drugs.

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Appendix #5

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Cisplatin: mode of cytotoxic action and molecular basis of resistance

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Cisplatin is one of the most potent antitumor agents known, displaying clinical activity against a wide variety of solid tumors. Its cytotoxic mode of action is mediated by its interaction with DNA to form DNA adducts, primarily intrastrand crosslink adducts, which activate several signal transduction pathways, including those involving ATR, p53, p73, and MAPK, and culminate in the activation of apoptosis. DNA damage-mediated apoptotic signals, however, can be attenuated, and the resistance that ensues is a major limitation of cisplatin-based chemotherapy. The mechanisms responsible for cisplatin resistance are several, and contribute to the multifactorial nature of the problem. Resistance mechanisms that limit the extent of DNA damage include reduced drug uptake, increased drug inactivation, and increased DNA adduct repair. Origins of these pharmacologic-based mechanisms, however, are at the molecular level. Mechanisms that inhibit propagation of the DNA damage signal to the apoptotic machinery include loss of damage recognition, overexpression of HER-2/neu, activation of the PI3-K/Akt (also known as PI3-K/PKB) pathway, loss of p53 function, overexpression of antiapoptotic bcl-2, and interference in caspase activation. The molecular signature defining the resistant phenotype varies between tumors, and the number of resistance mechanisms activated in response to selection pressures dictates the overall extent of cisplatin resistance.

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Keywords: cisplatin; mode of action; drug resistance; mechanism

Introduction

Since its introduction into clinical trials, cisplatin (*cis*-diammine-dichloro-platinum^{II}) has had a major impact in cancer medicine, changing the course of therapeutic management of several tumors, such as those of the ovary, testes, and the head and neck (Prestayko *et al.*, 1979). Almost 30 years after its clinical benefits were first recognized, studies still continue in an effort to understand exactly how cisplatin works. There is no doubt, however, that DNA is the primary target of cisplatin (Roberts and Pera Jr, 1983), but still there are wide gaps in our fuller appreciation of the process that translates cisplatin-induced DNA damage into its characteristic drug-mediated cellular effects, namely, inhibition of DNA synthesis, suppression of RNA transcription, effects on the cell cycle, and the therapeutically beneficial process of apoptosis. An understanding of the mode of action is indeed desirable in refining therapeutic approaches that further enhance the antitumor activity of the platinum drug. This understanding is also critical for elucidating mechanisms underlying the drug-resistant phenotype, which radically limits the clinical utility of cisplatin. An excellent example to highlight this limitation is with ovarian cancer, which generally responds well to cisplatin-based therapy. Unfortunately, the initial response rate of up to 70% is not durable, and results in a 5-year patient survival rate of only 15–20%, primarily as tumors become resistant to therapy (Ozols, 1991). In an alternative example with small cell lung cancer, the relapse rate can be as high as 95% (Giaccone, 2000). The onset of resistance creates a further therapeutic complication in that tumors failing to respond to cisplatin are cross-resistant to diverse unrelated antitumor drugs (Ozols, 1992). This suggests that cisplatin and the other agents likely share common mechanisms of resistance. In this respect, it is noteworthy that cisplatin-resistant tumors are fully crossresistant to the platinum analog carboplatin (Gore *et al.*, 1989; Eisenhauer *et al.*, 1990). Thus, to circumvent resistance, alternative DNA damage-signaling pathways need to be evoked, as has been demonstrated experimentally with ionizing radiation and the platinum analog DACH-acetato-Pt (Hagopian *et al.*, 1999; Siddik *et al.*, 1999). It is indeed likely that the demonstration of increased sensitivity of resistant cells to distinct platinum drugs, such as ZD0473 (Kelland *et al.*, 1999) and oxaliplatin (Faivre *et al.*, 1999) may in part reflect activation of independent pathways. Utilization of such agents in comparative investigations may prove to be invaluable for unraveling fully the mechanism of cisplatin resistance.

Mode of drug action

The pathways involved in cisplatin-induced cytotoxicity are summarized in Figure 1, and described in detail in the following sections.

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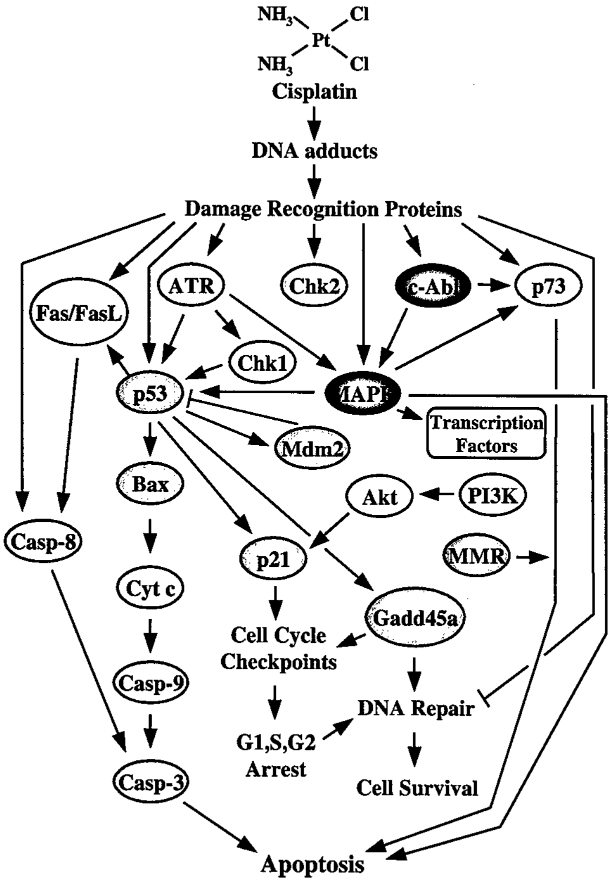

Figure 1 An overview of pathways involved in mediating cisplatin-induced cellular effects. Cell death or cell survival will depend on the relative intensity of the signals generated and the crosstalk between the pathways involved. Some of the signaling discussed in the text has been omitted for clarity

Drug reactivity

Cisplatin is a neutral inorganic, square planar complex that reacts with DNA to induce its characteristic biological effects, which culminate in either repair of the DNA damage and cell survival or activation of the irreversible apoptotic program. However, for interaction to occur with DNA, the neutral cisplatin has to be activated through a series of spontaneous aquation reactions, which involve the sequential replacement of the *cis*-chloro ligands of cisplatin with water molecules (el Khateeb *et al.*, 1999; Kelland, 2000). The mono-aquated form is recognized as a highly reactive species, but its formation is rate limiting in the interaction with many endogenous nucleophiles, such as glutathione (GSH), methionine, metallothionein, and protein. Thus, when cisplatin enters cells, it is potentially vulnerable to cytoplasmic inactivation by these and other intracellular components.

DNA adducts and damage recognition

The cytotoxicity of cisplatin is primarily ascribed to its interaction with nucleophilic N7-sites of purine bases in DNA to form DNA–protein and DNA–DNA interstrand and intrastrand crosslinks (Eastman, 1987b). However, evidence strongly favors intrastrand adducts as lesions largely responsible for the cytotoxic action (Pinto and Lippard, 1985). This is consistent with the knowledge that 1,2-intrastrand ApG and GpG crosslinks are the major forms of DNA adducts, accounting for 85–90% of total lesions (Kelland, 1993). A similar preponderance of these intrastrand adducts has also been reported in cultured cells for the structurally distinct analog DACH-sulfato-platinum^{II} (Jennerwein *et al.*, 1989). This eliminates the possibility that the favorable cytotoxicity of such analogs against cisplatin-resistant tumor cells (Eastman, 1987a) is due to a qualitative or quantitative difference in DNA bases that are targeted.

Since intrastrand DNA adducts comprise the bulk of cisplatin-induced nuclear lesions, it is not surprising that a linear correlation has been found between gross levels of platinum bound to DNA and the extent of cytotoxicity (Fraval and Roberts, 1979; Roberts and Fraval, 1980). Although cisplatin affects DNA replication, no correlation exists between inhibition of DNA synthesis and cytotoxicity (Sorenson and Eastman, 1988). It is only recently that we have come to understand better the sequence of events extending from the formation of DNA adducts to the completion of the cytotoxic process, namely apoptosis. This sequence is likely initiated or facilitated following the recognition of DNA damage by over 20 individual candidate proteins, which bind to physical distortions in the DNA that are induced by the intrastrand platinum adducts (Bellon *et al.*, 1991). These damage recognition proteins include the hMSH2 or hMutSα component of the mismatch repair (MMR) complex, the nonhistone chromosomal high-mobility group 1 and 2 (HMG1 and HMG2) proteins, the human RNA polymerase I transcription 'upstream binding factor' (hUBF), and the transcriptional factor 'TATA binding protein' (TBP) (Donahue *et al.*, 1990; Fink *et al.*, 1998; Chaney and Vaisman, 1999). Whether a single protein or combinations of these are involved in sensing the damage is not clear. What is interesting is that a few of the proteins, exemplified by MMR and HMG1, demonstrate greater preference for cisplatin adducts than for adducts induced by distinct platinum analogs, such as the clinically active oxaliplatin and JM216 (Fink *et al.*, 1996; Chaney and Vaisman, 1999; Zdraveski *et al.*, 2002).

Although the likely role of DNA damage recognition proteins is to transduce DNA damage signals to downstream effectors, their biological relevance may not be limited to this function alone. The HMG1 protein, for instance, has been implicated in promoting cytotoxicity by first interacting with the DNA adduct and then shielding it from repair (Huang *et al.*, 1994). This action of HMG1 is supported by the finding that overexpression of this recognition protein by pre-exposure to estrogen sensitizes breast tumor cells to cisplatin (He *et al.*, 2000). Similarly, hUBF and TBP are involved in the initiation of transcription by RNA polymerase I, and it is feasible that cisplatin adducts sequester these factors at the damaged DNA sites, and prevent their participation in transcription (Jordan and Carmo-Fonseca, 2000). The resulting inhibition of transcription may itself serve as a trigger for transducing DNA damage signals. It appears reasonable to suggest, therefore, that each of the recognition proteins may initiate one or more specific events, so that DNA damage results in several seemingly unrelated biological effects. This is consistent with the understanding that adducts induced by cisplatin disrupt replication and transcriptional processes, but that such biological effects do not necessarily correlate directly with cell death (Jordan and Carmo-Fonseca, 2000). This can also be reconciled by the understanding that both pro-survival and pro-apoptotic signals are activated simultaneously following cisplatin exposure, and the relative intensity and/or duration of each is integrated downstream to determine the final fate of the cell.

Cell cycle checkpoints

The notion that cisplatin-induced DNA damage activates a number of pathways is borne out from several investigations. One of these pathways culminates in the activation of cell cycle checkpoints, which temporally induce a transient S-phase arrest, followed by inhibition of the Cdc2-cyclin A or B kinase to affect a durable G2/M arrest (Shi *et al.*, 1994; Shapiro and Harper, 1999; He and Siddik, 2001). Since the inhibitory effect of DNA adducts of cisplatin on the G1-phase cyclin-dependent kinases (CDKs) is a later event in the sequence of checkpoint activation (He and Siddik, 2001), and likely facilitated by the Cdk4 inhibitor $p16^{INK4A}$ (Shapiro *et al.*, 1998), significant accumulation of cells in the G1 phase is seen infrequently, largely because cells remain trapped in G2/M. The relationship between cell cycle arrest and cytotoxicity is complex and not fully deciphered. If anything, cell cycle arrest is seen as inhibitory to the cytotoxic process, which is a conclusion that derives primarily from the demonstration that pharmacological abrogation of the G2/M checkpoint increases cellular sensitivity to cisplatin (Demarcq *et al.*, 1994; O'Connor and Fan, 1996). This is consistent with the concept that cell cycle arrest, as a generally accepted consequence of DNA damage, is necessary to enable the nucleotide excision repair (NER) complex to remove the adducts and promote cell survival. Only when repair is incomplete, as would be the case when damage is extensive, will cells undergo apoptosis. Thus, repair is intimately linked to checkpoint activation and apoptosis, and it is interesting that all three processes are collectively associated with the tumor-suppressor p53 protein (Morgan and Kastan, 1997; Bullock and Fersht, 2001). It is evident that our understanding of cellular and molecular responses to DNA-damaging agents has increased substantially during the past few years, but many important questions remain, including how p53 senses the extent of DNA damage repair and, thereby, determines whether to permit the cell to survive or activate the apoptotic program.

Activation of p53 and MAPK

Although the mediation of p53 in the cellular toxic effects of cisplatin is a direct consequence of DNA damage, a number of events must first occur to induce and activate the p53 protein molecule. A known upstream event is activation of kinases that regulate the stability and transcriptional activity of the p53 tumor suppressor. Among the two kinases involved in checkpoint activation, namely ATM (ataxia telangiectasia mutated protein) and ATR (ATM- and Rad3-related protein), cisplatin preferentially activates ATR kinase (Damia *et al.*, 2001; Zhao and Piwnica-Worms, 2001), which phosphorylates p53 at serine-15 to initiate activation of the p53 protein (Appella and Anderson, 2001). ATR also activates other downstream targets as a step toward further modification of p53 at additional sites. Thus, ATR-mediated activation of CHK1 kinase results in phosphorylation at serine-20 of p53 (Shieh *et al.*, 2000). Interestingly, cisplatin also activates CHK2, which is a downstream target of ATM, but the effect of cisplatin on CHK2 appears to be independent of ATM (Damia *et al.*, 2001). More recently, ATR has been linked to the activation of specific pathways of the mitogen-activated protein kinase (MAPK) cascade (Tang *et al.*, 2002; Zhang *et al.*, 2002), which phosphorylates p53 in a number of positions, including serine-15 (Persons *et al.*, 2000) and threonine-81 (Appella and Anderson, 2001).

The involvement of the MAPK pathway in cisplatin's mode of action is of significant interest. The major MAPK subfamily members include the extracellular signal-regulated kinases (ERK), the c-Jun N-terminal kinases (JNKs, also referred to as stress-activated protein kinase (SAPK)), and the p38 kinases. These MAPK members participate in integrating extracellular signals to regulate cell proliferation, differentiation, cell survival, and apoptosis (Dent and Grant, 2001). Studies by Wang *et al.* (2000) have demonstrated that all three kinase members are activated following exposure of tumor cells to cisplatin. These authors, however, suggest that ERK activation is the most critical for cisplatin-induced apoptosis, which is consistent with the demonstration that ERK activated by cisplatin contributes to p53 regulation by phosphorylating the tumor-suppressor protein at serine-15 (Persons *et al.*, 2000). Furthermore, inhibition of the MEK–ERK pathway leads to cisplatin resistance (Yeh *et al.*, 2002). Reports by others, however, are in direct contrast and suggest that activation of ERK and JNK MAPK cascades by cisplatin antagonizes apoptosis (Dent and Grant, 2001). It is possible that both effects mediated through MAPK are correct, and the apparent discrepancy may merely reflect differences in cell context or the extent of DNA damage. Thus, it may be premature at this stage to disassociate any MAPK subfamily members from the cytotoxic effects of cisplatin.

p53-dependent functions

Induction and/or activation of p53 is recognized as a prerequisite for its function as a sequence-specific transcription activator. Interestingly, HMG1 and HMG2 facilitate the binding of p53 to DNA to stimulate transactivation, and this enables HMG proteins to establish a direct link between damage recognition and activation of p53 function (Jayaraman *et al.*, 1998). Several genes transactivated by p53 as a result of cisplatin exposure are associated with cell cycle arrest, DNA repair, and apoptosis, including CDK inhibitor $p21^{Waf1/Cip1}$, growth arrest, and DNA damage-inducible *gadd45a* gene, and the pro-apoptotic *bax* gene (Delmastro *et al.*, 1997; Hershberger *et al.*, 2002). The p53 protein can also transactivate *mdm2*, which is a negative feedback regulator of p53 activity (Alarcon-Vargas and Ronai, 2002). With regard to repair, the Gadd45a protein associates with proliferating cell nuclear antigen (PCNA), enhances NER activity, and protects cells from cisplatin-induced cytotoxicity (Smith *et al.*, 1994; Delmastro *et al.*, 1997; Smith *et al.*, 1997). However, when DNA damage exceeds a critical threshold, and presumably overwhelms cellular repair capacity, the net biological effect favors activation of apoptosis. This form of cell death is a complex, well-orchestrated process that begins with the translocation of the cisplatin-induced Bax from the cytosol to the mitochondria, where a cascade of events, involving the release of apoptogenic factors (such as cytochrome *c*) activates the caspase 9–caspase 3 pathway, and results in apoptosis (Wang *et al.*, 2000; Makin *et al.*, 2001). More specifically, the apoptotic process is regulated by the ratio between Bax and its opposing but closely related antiapoptotic counterpart Bcl-2. When Bax is induced by cisplatin, the Bax : Bcl-2 ratio increases and apoptosis ensues. However, cisplatin may also induce cleavage of Bcl-2, and either the resultant Bax-like cleaved product or the effective increase in the Bax : Bcl-2 ratio activates the apoptotic cascade (del Bello *et al.*, 2001). Apoptosis induced by cisplatin also occurs through the Fas/FasL-activated caspase 8–caspase 3 pathway, which is facilitated by p53 function, but does not necessarily involve the mitochondria (Micheau *et al.*, 1997; Muller *et al.*, 1998). However, this pathway is not well understood as caspase 8 or apoptosis can be activated by cisplatin independent of Fas/FasL in some systems (Eischen *et al.*, 1997; Ferreira *et al.*, 2000).

Q5

Induction of apoptosis

Although the propensity of the reported data supports a facile role for p53 in cisplatin-induced apoptosis (Fan *et al.*, 1994; Segal-Bendirdjian *et al.*, 1998), there are several reports that deviate from this understanding. Fan *et al.* (1995) and Hawkins *et al.* (1996), for instance, have demonstrated that disruption of p53 function sensitizes tumor cells to the platinum drug, and do not make them resistant, as would be expected. It is useful to note that this counterintuitive finding is associated with tumor cells that appear to have an apoptotic dysfunction (Fan *et al.*, 1995). How eliminating p53 function makes such cells more sensitive to cisplatin is unclear, but it is likely that cell cycle effects come into play, since sensitization to cisplatin is mediated through down-regulation of the p53-dependent $p21^{Waf1/Cip1}$ gene (Fan S *et al.*, 1997). The increased sensitivity to cisplatin in such cases may be ascribed to a loss in the contributory role of $\mathrm{p21^{Waf1/Cip1}}$ in G2/M arrest, resulting in premature entry into mitosis, with cell death being the final outcome. Such an effect is analogous to the observed sensitization of tumor cells to cisplatin by agents that abrogate the G2/M checkpoint (O'Connor and Fan, 1996). A further demonstration of the ability of cisplatin to induce cytotoxicity through a mechanism not involving p53 comes from the work of Gong *et al.* (1999), who reported that the protein product of a p53-related gene, *p73*, can also be induced by cisplatin to mediate apoptosis. Indeed, this group has demonstrated the coexistence of p53- and p73-dependent parallel apoptotic pathways for affecting cisplatin-induced cytotoxicity. Induction of p73-dependent apoptosis by cisplatin has two requirements: (1) drug-activated c-Abl tyrosine kinase and (2) cellular proficiency of the MMR complex, which, as with HMG1, links damage recognition to apoptotic signaling. c-Abl activated by cisplatin can also upregulate the MEKK–MKK–JNK pathway (Kharbanda *et al.*, 2000), but the implied association between this specific MAPK pathway and p73 has been uncertain previously. However, the case for this association has been strengthened by recent evidence, which shows that activation of p73 by c-Abl also requires the activity of p38 as a representative of the MAPK subfamily member (Sanchez-Prieto *et al.*, 2002).

Mechanism of resistance

The major goal of cancer chemotherapy is to commit tumor cells to apoptosis following exposure to antitumor agents. Although the inorganic drug cisplatin is a very potent inducer of apoptosis (Ormerod *et al.*, 1996; Henkels and Turchi, 1997), resistance develops and is implied when tumor cells fail to undergo apoptosis at clinically relevant drug concentrations. This resistance can be acquired through chronic drug exposure or it can present itself as an intrinsic phenomenon. The exact level of cisplatin resistance in patients is difficult to define, but at least a twofold resistance is inferred from clinical studies, primarily since responses have been observed when the standard clinical dose of cisplatin is doubled in drug-intensive therapy protocols (Ozols *et al.*, 1984, 1988; Schilder and Ozols, 1992). In general, resistance to cisplatin may be substantially greater, as judged from studies with tumor cell lines established from clinically refractory tumors, which require cytotoxic concentrations as much as 50–100-fold in excess of those needed for sensitive tumor cells (Hills *et al.*, 1989; Kelland *et al.*, 1995; Hagopian *et al.*, 1999). Thus, the problem posed by cisplatin resistance appears to be more severe than has been acknowledged in the past. It should be noted that although mechanisms of resistance have largely been derived from tissue culture studies, there is good evidence for a general agreement with mechanisms encountered clinically (Giaccone, 2000).

With the understanding that the cytotoxic effect of cisplatin is a complex process, extending from initial drug entry into cells to the final stages of apoptosis (see Figure 1), it follows that intracellular events interfering with any stage of this process will inhibit apoptosis and lead to drug resistance. Resistance mechanisms, therefore, arise as a consequence of intracellular changes that either prevent cisplatin from interacting with DNA, interfere with DNA damage signals from activating the apoptotic machinery, or both. Substantial evidence exists to indicate that the level and persistence of DNA adducts induced by cisplatin correlate directly with cytotoxicity (Fraval and Roberts, 1979; Roberts and Fraval, 1980). Reducing the extent of DNA damage, therefore, increases resistance, and this can occur through changes in drug accumulation, intracellular thiol levels, and/or DNA adduct repair. Thus, a reduction in the level of DNA adducts is generally ascribed to biochemical/molecular pharmacologic alterations, which are secondary to primary genetic changes. On the other hand, interference in initiating or transducing damage signals to inhibit apoptotic activation is due to changes at the molecular biologic/genetic level. Although a single mechanism of cisplatin resistance in a tumor cell is possible (Kelland *et al.*, 1992b), in practice it is extremely rare. In general, resistance is multifactorial, in that several mechanisms are encountered simultaneously within the same tumor cell (Richon *et al.*, 1987; Teicher *et al.*, 1987; Eastman *et al.*, 1988). Thus, the high level of resistance is a net effect of several unrelated mechanisms (Siddik *et al.*, 1998), which compounds the difficulty in efforts to circumvent cisplatin resistance as a therapeutic strategy.

The specific mechanisms involved in cisplatin resistance are several, and discussed below in detail.

Reduced intracellular drug accumulation

There is ample evidence to indicate that reduced drug accumulation is a significant mechanism of cisplatin resistance. Reductions of the order of 20–70% have been documented in a variety of cell lines displaying resistance to cisplatin by a factor of 3–40-fold (Kelland, 1993). As expected from consideration of the multifactorial nature of the resistance mechanism, reduction in drug accumulation is not directly proportional to the level of resistance (Johnson *et al.*, 1997). Indeed, the profile of resistance mechanisms of a given tumor cell line may not include defects in drug accumulation as a mechanism (Teicher *et al.*, 1991; Kelland *et al.*, 1992b). On the other hand, in some cancer cells, reduction in cisplatin accumulation is the principal mechanism of resistance, accounting for 70–90% of total resistance (Kelland, 1993).

The cause of the reduced cisplatin accumulation in resistant cells may be ascribed to either an inhibition in drug uptake, an increase in drug efflux, or both. A defect in the uptake process appears to be prevalent, but the mechanism for this remains obscure. Since reduced uptake can be demonstrated over a wide range of extracellular cisplatin concentrations, it is likely that resistance occurs as a result of changes in the nonsaturable process of passive drug diffusion (Yoshida *et al.*, 1994; Kelland, 2000). There is limited evidence, however, that an energy-dependent active transport involving Na^+K^+-ATPase or a gated ion channel has a role in cisplatin uptake (Andrews *et al.*, 1988; Gately and Howell, 1993), and, therefore, an alteration in this system as a causative factor in cisplatin resistance cannot be totally ruled out.

Development of resistance as a result of increased cisplatin efflux was largely discounted in earlier studies (Teicher *et al.*, 1987; Andrews *et al.*, 1988). More recently, there has been a resurgence of interest in this resistance mechanism as new exporter proteins have been identified. The multidrug resistance-associated (MRP) gene family, composed of at least seven members (MRP1–7), has been a major target of investigations, primarily as several of these ABC membrane proteins have been found in tumor cells and associated with cellular efflux of a variety of drugs (Borst *et al.*, 2000). However, only MRP2 (cMOAT) appears to be important in cisplatin resistance, and this is consistent with the observation that resistant cells have increased levels of this transporter protein (Kool *et al.*, 1997). Moreover, a 10-fold increase in resistance has been demonstrated in cells overexpressing *MRP2* following gene transfection (Cui *et al.*, 1999). Support for the involvement of MRP2 in resistance also comes from the converse demonstration that transfection of tumor cells with an MRP2 antisense expression vector increases sensitivity to cisplatin (Koike *et al.*, 1997). It is useful to note that MRP2 is not universally associated with cisplatin resistance (Shen *et al.*, 2000). A second important area of investigation involving cisplatin efflux has centered around *ATP7A* and *ATP7B*, two copper-transporting P-type ATPase genes that are overexpressed in cisplatin-resistant tumor cells (Komatsu *et al.*, 2000; Katano *et al.*, 2002). More convincing has been the demonstration that human tumor cells transfected with *ATP7B* acquire significant resistance to both cisplatin (ninefold) and copper (twofold), primarily as a consequence of enhanced cisplatin efflux. The recent proposal to use overexpression of *ATP7B* as a clinical marker of chemoresistance to cisplatin in ovarian cancer affirms the potentially significant role of the copper transporter in cisplatin resistance (Nakayama *et al.*, 2002).

Independent studies to involve either the multidrug resistance (MDR) P-glycoprotein pump (Smith *et al.*, 1993; Wada *et al.*, 1999; Bible *et al.*, 2000) or the major vault/lung resistance-related protein (MVP/LRP) transporter directly (Mossink *et al.*, 2002) in cisplatin efflux have been largely inconclusive. Caution, however, needs to be exercised since a clinical study in advanced ovarian cancer using a cisplatin-based treatment regimen has demonstrated that P-glycoprotein overexpression is associated with a poor chemotherapeutic outcome (Baekelandt *et al.*, 2000). Similarly, advanced ovarian cancers having increased levels of MVP/LRP respond poorly to cisplatin (Izquierdo *et al.*, 1995). It is apparent that further studies are needed to clarify and/or amplify the roles of P-glycoprotein and MVP/LRP in cisplatin resistance.

Increased inactivation by thiol-containing molecules

The much lower chloride concentration (~4 mmol/l) in the cytoplasm facilitates aquation reactions, which activate cisplatin and enable it to react with, and become inactivated by a number of cytoplasmic constituents, including the abundant nucleophilic GSH and the cysteine-rich metallothionein. Concentrations of these thiol-containing molecules increase following chronic cisplatin exposure, and induce resistance by decreasing the level of the antitumor agent available for interaction with the target DNA. Inactivation of cisplatin by GSH and pathways promoting this reaction are shown in Figure 2.

Increases in GSH have been demonstrated in a number of cisplatin-resistant tumor models (Kelland, 1993), and confirmed in clinical studies (Wolf *et al.*, 1987). Furthermore, in a panel of resistant ovarian tumor models, prominent elevations in GSH levels have been correlated directly with resistance. Such elevations may occur as a result of increased expression of the γ-glutamylcysteine synthetase (γ-GCS) gene (Mistry *et al.*, 1991; Godwin *et al.*, 1992; Hamaguchi *et al.*, 1993), the translational product of which is a rate-limiting enzyme involved in GSH biosynthesis (Figure 2). These changes in GSH and γ-GCS appear to be mediated through upregulation of the transcription factor c-Jun (Pan *et al.*, 2002). Resistance due to elevated GSH, however, is reversible and parallels the decline in this thiol molecule when cisplatin is removed from cell cultures (Hamaguchi *et al.*, 1993). An increase in GSH following chronic cisplatin exposure, however, is not a general occurrence, and this likely contributes to the negative correlation in some studies between GSH levels and cisplatin sensitivity (D'Incalci *et al.*, 1998; Kolfschoten *et al.*, 2000).

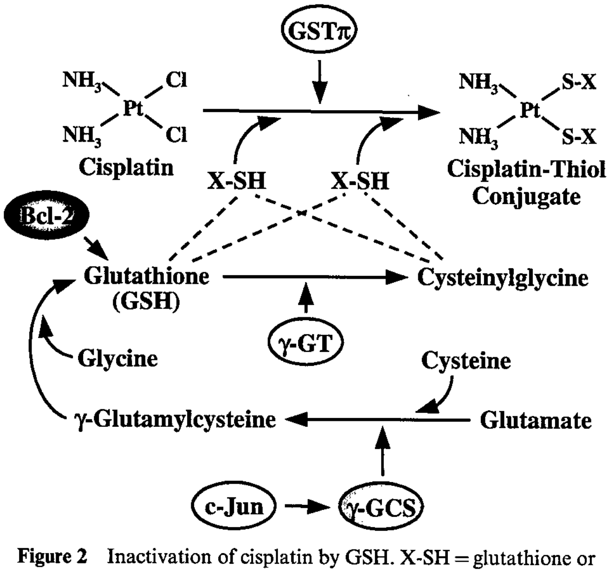

Figure 2 Inactivation of cisplatin by GSH. X-SH = glutathione or cysteinylglycine

The high reactivity of aquated cisplatin promotes its interaction with GSH in a nonenzymatic manner. This conjugation reaction, however, can also be catalysed by GSH-S-transferase π (GSTπ), which is a member of a family of enzymes involved in xenobiotic detoxication reactions (Goto *et al.*, 1999). The increased expression of GSTπ (Sakamoto *et al.*, 2001), together with elevated GSH levels in resistant tumor cells, suggests that enzymatic inactivation of cisplatin contributes significantly to the resistance phenotype at the clinical level. Indeed, a low level of GSTπ has been correlated to an overall survival rate of 82% with cisplatin in head and neck cancer patients, whereas a high level of the enzyme was associated with a twofold reduction in survival (Shiga *et al.*, 1999). Overexpression of γ-glutamyltransferase (γ-GT) in cisplatin resistance is also observed, and this may further exacerbate inactivation of cisplatin (Daubeuf *et al.*, 2002). γ-GT is a key player in GSH homeostasis, and generates cysteinylglycine during GSH catabolism (Figure 2). Since cysteinylglycine is 10-fold more reactive toward cisplatin than is GSH, the overproduction of the more reactive thiol by γ-GT is potentially a major contributor to GSH-mediated resistance.

Undoubtedly, the increased conjugation reaction between GSH and cisplatin is generally accepted as a significant factor in resistance, but other explanations for the effect of GSH are also of interest. These include the role of elevated GSH in either increasing DNA repair (Kelland, 1993) or increasing the inhibitory effect on apoptosis by buffering an endogenous drug-induced oxidative stress (Chiba *et al.*, 1995; Slater *et al.*, 1995). This is consistent with reports that cells overproducing the Bcl-2 protein have correspondingly higher intracellular GSH levels, which may contribute to the anti-apoptotic functions of Bcl-2 (Hockenbery *et al.*, 1993; Chiao *et al.*, 1995).

Metallothioneins are rich in thiol-containing cysteine molecules, which also provide ideal reactive centers for interaction with cisplatin, in much the same way as with GSH. It is not unexpected, therefore, that increases in metallothionein, up to fivefold over basal levels, have been observed in cisplatin-resistant murine and human tumor models (Kelley *et al.*, 1988; Kasahara *et al.*, 1991). It is noteworthy that in some studies, changes in metallothionein levels in resistant cell lines, or in human ovarian tumor biopsies taken before and after cisplatin-based therapy, have not been observed (Andrews *et al.*, 1987; Schilder *et al.*, 1990; Murphy *et al.*, 1991). These variations in the reported data again emphasize the multifactorial nature of resistance and also that the increase in metallothionein is not necessarily an absolute requirement for cells to attain the resistance phenotype.

Increase in DNA damage repair

Formation and persistence of DNA adducts of cisplatin are vital in inducing apoptosis. Therefore, an enhanced rate of adduct repair will attenuate the apoptotic process. This is supported by the demonstration that an increased rate of repair is associated with an inhibition of drug-induced cytotoxicity in several murine and human tumor cell lines (Lai *et al*., 1988; Sheibani *et al*., 1989; Chao *et al*., 1991; Kelland *et al*., 1992a; Siddik *et al*., 1998). As with other mechanisms, repair is not universally present in all cisplatin-resistant cell lines (Schmidt and Chaney, 1993). When present, however, the contribution of increased repair to resistance is low, and usually results in resistance of the order of 1.5–2.0-fold. This limited increase is, nevertheless, considered as significant, and highlighted by the understanding that the inactivity of the transplatin congener is largely due to the rapid repair of its DNA adducts (Heiger-Bernays *et al*., 1990). The implied upper limit for repair capacity in resistance is supported by the finding that increased repair is unchanged even when resistance to cisplatin increases progressively in chronic drug exposure protocols (Chaney and Sancar, 1996; Eastman and Schulte, 1988). Moreover, topoisomerase II is linked to repair of cisplatin-induced DNA crosslinks, and it is not inconsistent to find that its overexpression in cases of clinical cancer is associated with the onset of cisplatin resistance (Ali-Osman *et al*., 1993; Hengstler *et al*., 1999). Factors contributing to enhanced repair are indicated in Figure 3.

NER is the major pathway for platinum adduct removal and repair of DNA damage. The significance of NER is highlighted by the finding that a cellular defect in this pathway results in hypersensitivity to cisplatin, and that restoration of NER integrity re-establishes sensitivity to normal levels (Chaney and Sancar, 1996; Furuta *et al*., 2002). NER has broad specificity, and no differences are observed in the excision of adducts induced by cisplatin and structurally diverse platinum-based drugs (Chaney and Vaisman, 1999). Indeed, enhanced repair of adducts in resistant cells also applies to platinum analogs that are effective against the resistance phenotype (Jennerwein *et al*., 1991), and this suggests that increased repair as a mechanism of resistance may be difficult to overcome through the platinum analog drug development process. Although the NER complex is composed of at least 17 different proteins (Sancar, 1994; Friedberg, 2001), it appears that upregulation of only a few rate-limiting proteins is necessary to increase the excision repair capacity in resistant tumor cells (Reed, 1998). For instance, cisplatin resistance is associated with increases in the excision repair crosscomplementing ERCC1 or ERCC1/XPF complexes, but not ERCC3 (Lee *et al*., 1993; Friedberg, 2001). This finding with ERCC1 is of clinical relevance, as a twofold increase in *ERCC1* mRNA levels has been noted in patient's tumors that have become insensitive to cisplatin (Dabholkar *et al*., 1994). Similarly, the NER-related *XPA* gene is also overexpressed in cisplatin resistance and contributes to enhanced repair (Dabholkar *et al*., 1994). Conversely, testicular tumor cells, which are highly sensitive to cisplatin, express very low levels of XPA and ERCC1/XPF (Koberle *et al*., 1999).

The NER complex is responsible for both global genomic and transcription-coupled nucleotide excision repair (TC-NER) of cisplatin-induced DNA adducts (Chaney and Sancar, 1996). An early signal for activation of the TC-NER pathway, which allows preferential repair of the transcribed strand of an active gene, is thought to be the stalling of RNA polymerase II at DNA helix-distorting lesions (Svejstrup, 2002). Several proteins, such as ERCC1 and XPA, play a key role in TC-NER, with ERCC1 demonstrating a preference for repairing interstrand platinum crosslinks in actively transcribed genes, such as the dihydrofolate reductase (*DHFR*) gene; ERCC1-mediated TC-NER of intrastrand lesions in *DHFR* gene is either inefficient or unchanged in resistant cells (Larminat and Bohr, 1994; Chaney and Sancar, 1996). Since intrastrand adducts are the critical cytotoxic lesions of cisplatin, and since assay techniques for gene-specific repair of interstrand crosslinks have been questioned, the significance of TC-NER in cisplatin resistance is considered by some as doubtful (Chaney and Sancar, 1996). This, however, is countered by the compelling demonstration that breast and ovarian cancer susceptibility gene *BRCA1* is involved in TC-NER (Gowen *et al*., 1998), and that overexpression or inhibition of this gene is associated with cisplatin resistance or sensitivity, respectively (Husain *et al*., 1998). Furthermore, cells deficient specifically in TC-NER are hypersensitive to cisplatin (Furuta *et al*., 2002).

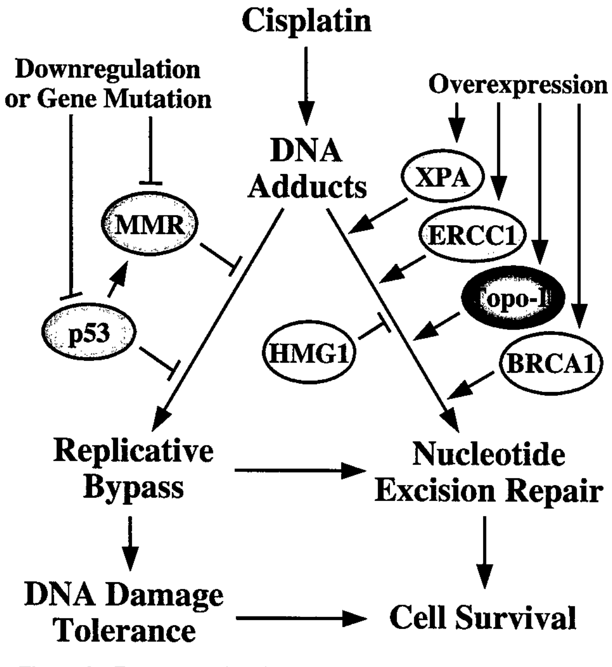

Figure 3 Factors modulating repair of cisplatin-induced DNA adducts and regulating replicative bypass

Before repair is initiated, the damage to the DNA has to be recognized by specific proteins. Indeed, a number of DNA damage recognition proteins have been identified, but studies to define their involvement in cisplatin-resistant tumor cells have largely been confined to the MMR complex. It is noteworthy that MMR serves a critical role in maintaining the integrity of the genome through repair of DNA mismatch lesions, but it does not actually repair cisplatin adducts. A proposed viewpoint is that MMR attempts to repair the lesion, but in failing to do so activates the apoptotic signal (Vaisman *et al.*, 1998). The MMR complex consists of a number of proteins, including hMSH2, hMSH6, hMLH1, hMutLα (heterodimer of hMLH1 and PMS2), and hMutSα (a heterodimer of hMSH2 and hMSH6), with hMSH2 and hMutSα involved directly in recognizing GpG intrastrand adducts of cisplatin (Duckett *et al.*, 1996; Mello *et al.*, 1996; Fink *et al.*, 1998; Vaisman *et al.*, 1998; Zdraveski *et al.*, 2002). It is not surprising, therefore, that downregulation or mutations in MMR genes *hMLH1* or *hMSH2* are observed consistently in cisplatin resistance (Aebi *et al.*, 1996; Drummond *et al.*, 1996; Fink *et al.*, 1996; Brown *et al.*, 1997; Vaisman *et al.*, 1998). Interestingly, loss of MMR in cisplatin resistance is associated with microsatellite instability and reduced apoptosis (Anthoney *et al.*, 1996; Mayer *et al.*, 2002). From the viewpoint of relevance, the level of resistance induced by the loss in MMR is about 2–5-fold, which is clinically significant. In contrast to the deficiency of MMR in cisplatin resistance, the alternative recognition protein HMG1 is overexpressed in resistant tumor cells (Nagatani *et al.*, 2001). HMG1 is reported to shield DNA adducts from repair and its overexpression has been associated with cisplatin sensitivity (He *et al.*, 2000), so the significance of increased levels of HMG1 in cisplatin resistance is not presently known.

In order to ensure genomic stability, it is vital that repair of DNA occurs prior to DNA replication. However, resistance arises when cells enhance their capacity to replicate DNA past the adduct, and then initiate postreplication repair (Chaney and Sancar, 1996). This in essence increases the ability of tumor cells to tolerate high levels of DNA adducts induced by cisplatin (Figure 3). In this respect, it is significant that replicative bypass is increased 3–6-fold by defects in hMLH1 or hMSH6, which attaches further importance to the role of MMR in cisplatin resistance (Vaisman *et al.*, 1998). However, increased replicative bypass may also occur independent of MMR (Mamenta *et al.*, 1994). It is noteworthy that increased tolerance to DNA adducts is not only seen in MMR deficiency but can also occur following p53 malfunction (see below). Indeed, p53 dysfunction exacerbates cisplatin resistance in MMR-deficient tumor cells (Lin *et al.*, 2000, 2001), and this is consistent with both a downregulation of *hMSH2* by mutant p53 protein and an enhanced replicative bypass (Scherer *et al.*, 1996). Moreover, loss of the p53 function accompanies MMR deficiency in cell lines selected for cisplatin resistance (Anthoney *et al.*, 1996). Disruptions in crosstalks, as exemplified here between p53 and MMR, are probably at the center of the highly resistant phenotype.

Overexpression of HER-2/neu and the PI3-K/Akt pathway

The *HER-2/neu* proto-oncogene encodes a transmembrane receptor tyrosine kinase of 185 kD (p185), which has extensive homology to the epidermal growth factor receptor (EGFR) (Bargmann *et al.*, 1986; Yamamoto *et al.*, 1986). A poor response of human cancers to cisplatin is associated with amplification and overexpression of *HER-2/neu*, found in about 20–30% of breast and ovarian cancer patients (Slamon *et al.*, 1989; Hengstler *et al.*, 1999). Cisplatin resistance is similarly observed in model systems following transfection of tumor cells with an *HER-2/neu* expression vector (Tsai *et al.*, 1995). Conversely, suppression of p185 activity by the tyrosine kinase inhibitor emodin or an antibody to the HER-2/neu receptor potentiates cisplatin cytotoxicity, which may in fact be mediated by a reduction in cisplatin-DNA adduct repair (Pietras *et al.*, 1994; Zhang and Hung, 1996). However, contradictory results have also been observed in a few cases, as exemplified by an increase in cisplatin potency following induction of p185 tyrosine phosphorylation activity (Arteaga *et al.*, 1994).

Once the HER-2/neu receptor is activated, downstream signaling is propagated through either the SHC/GRB2/SOS pathway, which in turn activates the Ras/MAPK pathway (see below), or the PI3-K/Akt pathway (Hung and Lau, 1999). Basal activity of the PI3-K/Akt pathway facilitates the induction of p21$^{\mathrm{Waf1/Cip1}}$ by cisplatin in a p53-dependent manner, but without necessarily modulating Bax expression (Mitsuuchi *et al.*, 2000). In contrast, *HER-2/neu* overexpression enhances the activity of Akt, which associates with p21$^{\mathrm{Waf1/Cip1}}$ and phosphorylates the latter at threonine-145, thereby ensuring cytoplasmic localization of the CDK inhibitor (Zhou *et al.*, 2001). The resulting diminution in nuclear levels of p21$^{\mathrm{Waf1/Cip1}}$ by HER-2/neu overexpression may then explain the attenuation of cisplatin-mediated antiproliferative effects (Figure 4). Thus, p21$^{\mathrm{Waf1/Cip1}}$ function can be either promoted or attenuated by the PI3/Akt, depending on the strength of the upstream signal. In addition, Akt promotes the phosphorylation of the Mdm2 oncoprotein and its translocation into the nucleus, where Mdm2 downregulates the p53 tumor-suppressor protein to induce resistance (Mayo and Donner, 2002; Oren *et al.*, 2002; Zhou and Hung, 2002). The major cause for the onset of cisplatin resistance by HER-2/neu, however, may also be due to inactivation of the pro-apoptotic protein Bad following its phosphorylation by Akt (Hayakawa *et al.*, 2000). Phosphorylation of Bad by ERK MAPK at an alternative site similarly attenuates cisplatin cytotoxicity (Hayakawa *et al.*, 2000), and this may be exacerbated by HER-2/neu overexpression. To add to the complexity, the antiapoptotic signal may occur as a result of Akt-mediated phosphorylation of procaspase 9, which is then inactivated (Cardone *et al.*, 1998). Moreover, this antiapoptotic signaling to suppress cisplatin cytotoxicity

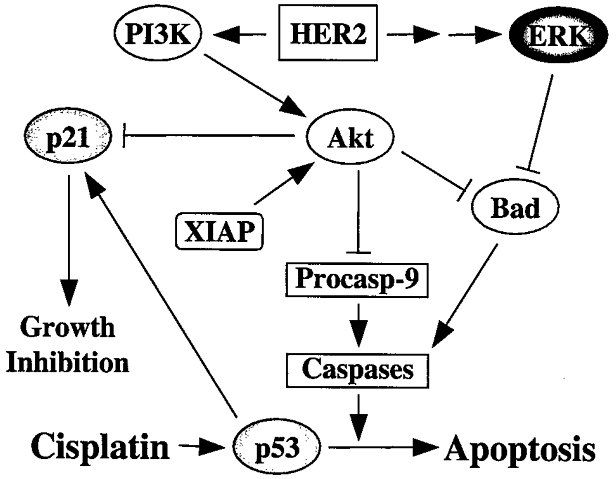

Figure 4 Cisplatin resistance affected through the HER-2/neu and PI3-K/Akt pathways

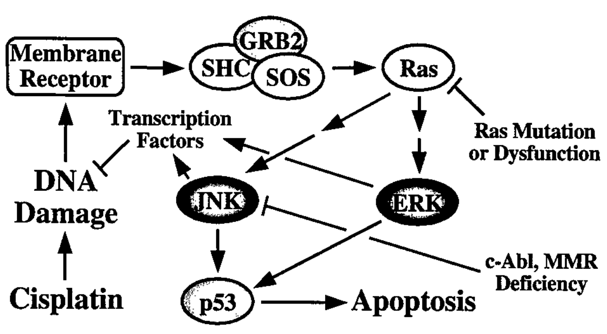

Figure 5 Attenuation of Ras and MAPK signaling pathways in cisplatin resistance

may include upregulation of Akt by XIAP (X-linked inhibitor of apoptosis protein) to facilitate inhibition of the caspase cascade (Asselin *et al.*, 2001). How the PI3-K/Akt and MAPK signals are integrated downstream to induce either cell survival or cell death is not well understood. Evidence is apparent, however, for intricate crosstalk between several pathways, including those involving Akt, p53, and Mdm2, and the relative intensity and/or duration of each activated pathway may determine the final fate of cells (Gottlieb *et al.*, 2002). Some of these pathways are depicted in Figure 4 (see also Figure 6).

Role of ras and MAPK pathway

As discussed earlier, MAPK subfamily members (p38, JNK, and ERK) are intimately associated with the mode of action of cisplatin. Whether a defect in the activation of MAPK pathway mediates cisplatin resistance is not clear, especially since some of the evidence points to both an increase and decrease in cisplatin sensitivity when the pathway is inhibited directly in human melanoma cells with PD98059, a specific MEK/ERK2 MAPK inhibitor (Mandic *et al.*, 2001). Moreover, the increased sensitivity is seen in both cisplatin-sensitive and -resistant cell lines, drawing the rational conclusion that cisplatin resistance may not be related to the JNK1 or ERK1/2 MAPK pathway (Cui *et al.*, 2000). Other studies, on the other hand, clearly establish the involvement of these pathways in mediating resistance, as is evident from studies utilizing the PD98059 inhibitor in a human cervical tumor cell system (Yeh *et al.*, 2002). Furthermore, resistance appears following perturbation of the pathway by dysfunction of the H-Ras proto-oncogene, which is an upstream activator of JNK and ERK MAPK (Woessmann *et al.*, 2002). This perturbation in the pathway is consistent with the finding that tumors expressing either *ras* mutation (Van't Veer *et al.*, 1988) or *ras* overexpression (Fan J *et al.*, 1997; Dempke *et al.*, 2000) are resistant to cisplatin. It is also useful to note that activation of MAPK pathway by *ras* overexpression may not necessarily alter the tumor cell sensitivity to cisplatin (Holford *et al.*, 1998). This inconsistency in the effect of *ras* overexpression on cisplatin resistance remains unexplained, as is the effect of the MEK/ERK2 MAPK inhibitor, but differences in the cellular context of the tumor models used in the reported studies are a good possibility.

When activated, the Ras/MAPK pathway contributes to post-translational modification of the tumor-suppressor/transcription activator p53 (Figure 5). In this regard, JNK MAPK activated via the MAP/ERK kinase kinase (MEKK1) phosphorylates p53, and a lack of this effect due to defective upstream activation of MEKK1 is the probable mechanism contributing to cisplatin resistance (Fuchs *et al.*, 1998; Gebauer *et al.*, 2000). The MAPK pathway also leads to the activation of a number of other transcription factors, such as c-Myc, c-Fos, and c-Jun (Robinson and Cobb, 1997; Martin-Blanco, 2000). These factors are overexpressed in cisplatin resistance, and their downregulation resensitizes tumor cells to the platinum drug (Kartalou and Essigmann, 2001; Pan *et al.*, 2002). Since c-Fos and c-Jun are components of the AP1 transcription complex, which induces a number of genes, including ERCC1, metallothionein, and GST (Dempke *et al.*, 2000), increased drug inactivation or DNA adduct repair will reduce DNA damage and provides a partial explanation for their effect in moderating cisplatin response. Similarly, c-Jun expression is closely linked to GSH levels (Pan *et al.*, 2002), which inactivates cisplatin and further supports a reduction in DNA damage as a mechanism of cisplatin resistance mediated by overexpression of transcription factors (Figure 5). Interestingly, c-Fos and/or c-Jun is induced by cisplatin in both sensitive and resistant cells (Delmastro *et al.*, 1997; Kartalou and Essigmann, 2001). These transcription factors, therefore, may act as both inhibitors and facilitators of apoptosis depending on the cell type and context (Leppa and Bohmann, 1999). Indeed, the levels of transcription factors are indirectly impacted by the functional status and effects of other molecular components on MAPK signaling. In this regard, it is noteworthy that JNK activity induced by cisplatin is substantially greater in tumor cells demonstrating MMR proficiency than MMR deficiency (Nehme *et al.*, 1997). Similarly, activation of c-Abl and p73 by cisplatin is necessary to facilitate apoptosis and is dependent not only on their wild-type gene status, but also on the cellular presence of hMLH1 and, therefore, the status of MMR (Nehme *et al.*, 1997; Gong *et al.*, 1999; Ono *et al.*, 2001). From these considerations, it is not surprising that there is a link between c-Abl and JNK, and that cells lacking c-Abl become resistant to cisplatin by losing their ability to activate JNK (Kartalou and Essigmann, 2001).

Dysfunction of tumor-suppressor p53

Stabilization and activation of wild-type p53 are critical for cisplatin-mediated apoptosis. Therefore, tumor cells that have defects in the apoptotic function of p53 fail to activate the cell death program and enable them to become tolerant to DNA damage, which is a feature characteristic of resistance caused by disruption in signal transduction pathways (Kastan *et al.*, 1991; Hartwell and Kastan, 1994; Pietenpol *et al.*, 1994; Siddik *et al.*, 1998, 1999). There is a significant body of evidence to indicate that tolerance to cisplatin adducts is of substantial significance in cisplatin resistance (Siddik *et al.*, 1998). Indeed, an excellent correlation exists between DNA damage tolerance and the level of resistance (Johnson *et al.*, 1997; Siddik *et al.*, 1998; Yoshida *et al.*, 1998). The ability to tolerate DNA adducts induced by the platinum agent is also seen clinically in a variety of tumor types, including those originating from the ovary and the head and neck (Marx *et al.*, 1998; Righetti *et al.*, 1999; Shiga *et al.*, 1999; Cabelguenne *et al.*, 2000).

A major factor affecting the loss of apoptotic function is *p53* gene mutation (see Figure 6), which is observed in about a half of all cancers (Hollstein *et al.*, 1991; Soussi, 2000). Interestingly, there appears to be a correlation between *p53* gene status and cisplatin response among cancers considered sensitive to cisplatin; greatest response is observed in seminomatous germ cell tumors, which harbor predominantly wild-type *p53*, and a relatively lower response rate is noted in ovarian, head and neck, and metastatic bladder cancers, which demonstrate a 40–60% p53 mutation frequency (Sarkis *et al.*, 1995; Houldsworth *et al.*, 1998; Cabelguenne *et al.*, 2000; Reles *et al.*, 2001). When mutation does occur, it is commonly observed in exons 4–9 of p53, and this disrupts the ability of the tumor suppressor to bind to DNA and transactivate p53-dependent genes. The inability to transactivate bax specifically, and thereby prevent increase in the Bax : Bcl-2 ratio, is likely a major factor in affecting the resistant phenotype (Perego *et al.*, 1996). It should be noted that many of the studies to define the impediment caused by mutant p53 have been conducted in tumor model systems. There is little doubt from several such studies that downregulation of the apoptotic process in tumor cells expressing mutant p53 is a major mechanism contributing to cisplatin resistance (Fan *et al.*, 1994; Eliopoulos *et al.*, 1995; Perego *et al.*, 1996; Gallagher *et al.*, 1997; Righetti *et al.*, 1999). Since mutant p53 disrupts cell cycle arrest in G1, which is also the phase in which tumor cells are most sensitive to cisplatin, resistance due to loss in p53 function may be mediated in part by disruption in cell cycle checkpoints (Shah and Schwartz, 2001). Although such effects of mutant p53 abound, several contradictions have contributed to confusions regarding the role of mutant p53 in cisplatin resistance. For instance, the NCI panel of cell lines demonstrates a wide range of overlapping responses to cisplatin for the group of wild-type and mutant p53 tumor models, with some mutant p53 models expressing exquisite cisplatin sensitivity (O'Connor *et al.*, 1997). These observations have also been documented in clinical cases, where tumors demonstrate either sensitivity or resistance to cisplatin irrespective of the *p53* gene status (Righetti *et al.*, 1996). Other similar counterintuitive observations, with mutant p53 promoting sensitivity to cisplatin (Fan *et al.*, 1995; Hawkins *et al.*, 1996), suggest that the cellular context is an important variable in drug response. Moreover, the presence of mutation in *p53* may not necessarily negate wild-type p53 functions (Siddik *et al.*, 1998). Since it is clear that the 5-year survival rate is significantly greater in patients with tumors expressing wild-type p53 than mutant p53 (van der Zee *et al.*, 1995), the central role of wild-type p53 in facilitating cisplatin cytotoxicity cannot be ignored.

A significant understanding to emerge from collective consideration of the reported studies is that cisplatin resistance occurs irrespective of *p53* gene status. However, the resistance observed in cells harboring wild-type p53 can be substantially greater than that observed in tumor cells having mutant or null *p53* status (Siddik *et al.*, 1998; Hagopian *et al.*, 1999). This resistance in wild-type p53 cells is attributed to downregulation of cisplatin-mediated induction of wild-type p53 and its

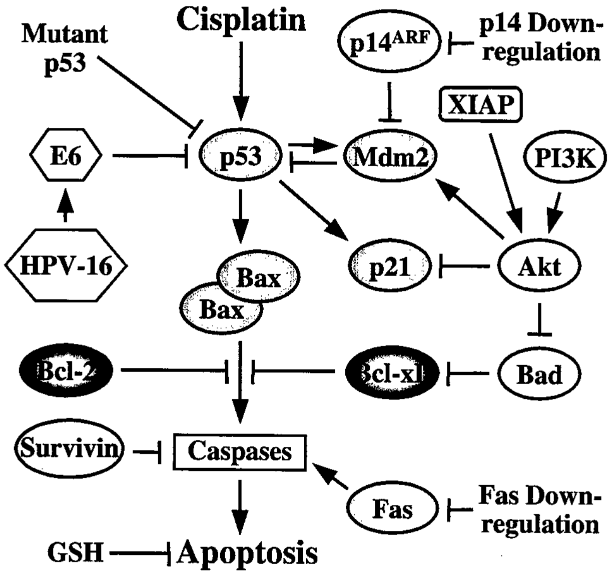

Figure 6 Disruption of p53-dependent apoptotic pathway in cisplatin-resistant tumor cells

inability to activate the apoptotic pathway (Figure 6). Intracellular factors that may inhibit such an activation of p53 include overexpression of the negative feedback regulator Mdm2 and downregulation of the moderator of Mdm2, p14^{ARF} (Fritsche *et al.*, 1993; Shieh *et al.*, 1997; Lakin and Jackson, 1999; Meek, 1999; Deng *et al.*, 2002). However, investigations to define their role in cisplatin resistance are limited, and conclusions on the involvement of Mdm2 in resistance are conflicting (Kondo *et al.*, 1995; Cocker *et al.*, 2001). Nevertheless, recent evidence suggests that p53 function can indeed be attenuated by Mdm2 through a pathway involving *HER-2/neu* overexpression and resultant activation of the PI3-K/Akt pathway (Mayo and Donner, 2002; Oren *et al.*, 2002; Zhou and Hung, 2002). The activity of wild-type p53 can also be attenuated by the human papillomavirus (HPV), which has been detected clinically in cancer of the cervix. In this case, the protein product of the *E6* oncogene in HPV-16 binds p53 to disrupt its transactivation and apoptotic functions, and causes platinum resistance (Kessis *et al.*, 1993; Hagopian *et al.*, 1999).

The apoptotic function of wild-type p53 is dependent on a number of cisplatin-induced upstream signaling pathways that stabilize and activate the tumor-suppressor protein by altering its phosphorylation and acetylation status (Fritsche *et al.*, 1993; Shieh *et al.*, 1997; Lakin and Jackson, 1999; Meek, 1999). It is not known, however, whether changes in these post-translational modifications of p53 affect resistance. The possibility that this may indeed occur is inferred from studies with a novel cisplatin analog that activates an independent DNA damage pathway to restore wild-type p53 function and, thereby, circumvent cisplatin resistance (Hagopian *et al.*, 1999; Siddik *et al.*, 1999).

Inhibitors of apoptosis

Molecular factors inducing cisplatin resistance do so by ultimately inhibiting apoptosis (see Figure 6). Apoptotic inhibitor molecules, such as survivin and XIAP, exacerbate resistance when overexpressed (Asselin *et al.*, 2001; Ikeguchi *et al.*, 2002). These inhibitors directly or indirectly impact the activities of caspases, which are the direct effectors of apoptosis, irrespective of the DNA damage pathway mediating the apotoic signal. For cisplatin, caspases 3, 8, and 9 are critical, and their activation is attenuated in resistant cells (Henkels and Turchi, 1999; Blanc *et al.*, 2000; Asselin *et al.*, 2001; Ono *et al.*, 2001). The inhibition of caspases 3 and 8 activation in these cells may be due in part to downregulation of the apoptotic signal as a result of a lack of *Fas* expression following cisplatin treatment (Qin and Ng, 2002).

Members of the Bcl-2 family are key players in regulating apoptosis (Farrow and Brown, 1996; Hanahan and Weinberg, 2000; Schuler and Green, 2001). They are localized in the mitochondria and have either pro- or antiapoptotic functions. The members form either homodimers or heterodimers, but only an excess level of homodimers can inhibit (e.g. Bcl-2/Bcl-2) or induce (e.g. Bax/Bax) apoptosis. The proapoptogenic Bax/Bax homodimer facilitates caspase activation through release of mitochondrial factors that include cytochrome *c* and Smac/DIABLO. This understanding is consistent with the requirement for p53-mediated transactivation of *bax* to affect cisplatin cytotoxicity (Eliopoulos *et al.*, 1995). In keeping with this understanding, overexpression of *bcl-2* is associated with cisplatin resistance, and this is likely facilitated by an increase in GSH levels (Hockenbery *et al.*, 1993; Chiao *et al.*, 1995) and compounded by the presence of mutant p53 (Strasser *et al.*, 1994; Herod *et al.*, 1996; Miyake *et al.*, 1999). Similarly, increased levels of the antiapoptotic protein Bcl-xL are also observed in resistant tumor cells (Gebauer *et al.*, 2000), possibly as a result of inhibition of the negative regulator Bad by the PI3-K/Akt pathway (Hayakawa *et al.*, 2000). Paradoxical findings, which indicate that *bcl-2* overexpression is associated with either improved survival of ovarian cancer patients receiving cisplatin (Herod *et al.*, 1996) or increased sensitivity of tumor cells to cisplatin (Beale *et al.*, 2000), serve to demonstrate our present limited knowledge of the highly complex apoptotic process.

Conclusion

Recently, we have witnessed a rapid expansion in our knowledge regarding molecular factors that not only play an intricate role in cisplatin's mode of action but

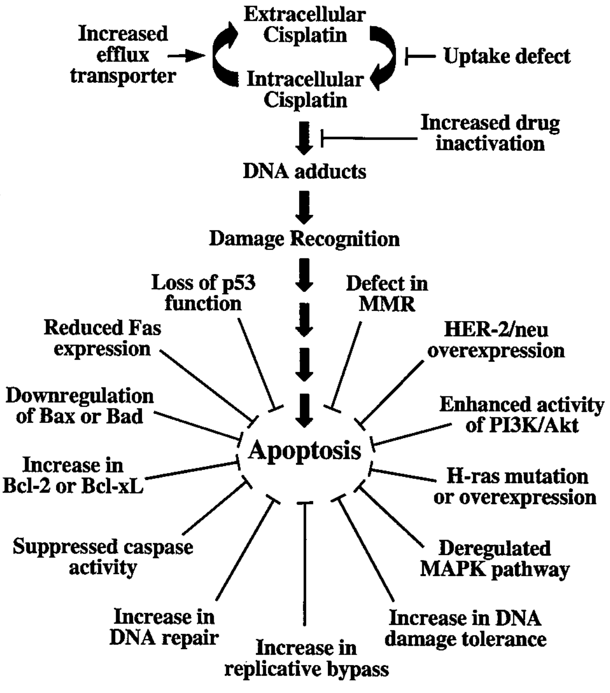
Extracellular Cisplatin
Increased efflux transporter
Uptake defect
Intracellular Cisplatin
Increased drug inactivation
DNA adducts
Damage Recognition
Loss of p53 function
Defect in MMR
Reduced Fas expression
HER-2/neu overexpression
Downregulation of Bax or Bad
Enhanced activity of PI3K/Akt
Apoptosis
Increase in Bcl-2 or Bcl-xL
H-ras mutation or overexpression
Suppressed caspase activity
Deregulated MAPK pathway
Increase in DNA repair
Increase in DNA damage tolerance
Increase in replicative bypass

Figure 7 Mechanisms involved in inhibiting the apoptotic signal in cisplatin-resistant tumor cells. More than one mechanism is usually observed in resistant cells, and this contributes to the multifactorial nature of cisplatin resistance

also impede the ability of the drug to induce apoptosis. Downregulation of the apoptotic signal is essentially a universal characteristic of resistance, and some of the mechanisms associated with cisplatin resistance and discussed in the preceding sections are summarized in Figure 7. However, there are still major gaps that need to be filled in order to understand fully the delicate interplay between molecular factors that promote either death of the cancer cell or survival of the resistant phenotype. The additional knowledge is essential if we are to devise future strategies to circumvent multifactorial mechanism of cisplatin resistance more effectively and, more importantly, to translate them into durable clinical responses.

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Appendix #6

Status of p53 Phosphorylation and Function in Sensitive and Resistant Human Cancer Models Exposed to Platinum-based DNA Damaging Agents

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Abstract

Purpose: Resistance to chemotherapeutic drugs is a hallmark of many human cancers, which can occur independent of p53 gene status. However, the presence of wild-type p53 in chemorefractory tumors confers greater resistance to cisplatin, but such tumors do not display complete cross-resistance to the platinum analog (1R,2R-diaminocyclohexane)-(trans-diacetato)(dichloro)platinum^{IV} (DACH-Ac-Pt). In this report, we have examined DNA damage-induced phosphorylation of p53 and downstream p53-dependent transactivation events in cisplatin-sensitive and -resistant human cancer cell lines possessing wild-type p53. *Methods:* Western-blot analysis was utilized to study the effect of cisplatin and the analog on p53 phosphorylation and p53-dependent target genes. *Results:* In response to CDDP and DACH-Ac-Pt, both CDDP-sensitive and -resistant models demonstrated a time- and dose-dependent inductions of total p53 protein and an increase in Ser-15 phosphorylation, which was more pronounced with CDDP. Although, phosphorylation of p53 at Ser-392 was also observed in CDDP-treated sensitive and resistant cells, it was weak or absent in response to DACH-Ac-Pt. Lack of Ser-392 phosphorylation by DACH-Ac-Pt, however, did not affect the induction of p21$^{WAF1/CIP1}$ or Mdm2. Similarly, inductions of p21$^{WAF1/CIP1}$ and Mdm2 were observed in sensitive cells exposed to cisplatin. In marked contrast, cisplatin-mediated induction of p21$^{WAF1/CIP1}$ was minimal or absent in resistant cells, but that of Mdm2 was unaffected. Wortmannin, a PI3-kinase (PI3-K) inhibitor, caused a dose-dependent inhibition of total p53 accumulation, Ser-15 phosphorylation and p21$^{WAF1/CIP1}$ transactivation in response to both CDDP and DACH-Ac-Pt, indicating that members of the PI3-K family are involved in phosphorylation of p53 and that transactivation of p21$^{\mathrm{WAF1/CIP1}}$ is p53-dependent. *Conclusion:* These studies demonstrate that cisplatin and DACH-Ac-Pt differentially phosphorylate p53 through independent DNA damage-induced pathways, and that the kinase-mediated phosphorylation of p53 at Ser-15 or Ser-392 is unaltered in resistance. Moreover, the phosphorylation status of Ser-392 on its own does not appear to correlate with p21$^{\mathrm{WAF1/CIP1}}$ or Mdm2 induction in these studies. However, a lack of increase in p21$^{\mathrm{WAF1/CIP1}}$ by cisplatin, but not DACH-Ac-Pt, provides a correlation with resistance and its circumvention, and implicates the role for cyclin-dependent kinase inhibitor in the differential cytotoxic effects of the two platinum agents against resistant cells.

Key words: p53 phosphorylation, cytotoxicity, p21$^{\mathrm{WAF1/CIP1}}$, drug resistance.

Introduction

Ovarian cancer is the fourth leading cause of cancer death among women in United States. In spite of cytoreduction and combination chemotherapy, the overall survival of the patients with advanced cancer is only 20-30% (Boente et al. 1993). Although aggressive treatment with platinum-based anti-tumor agents (CDDP and carboplatin) plays an important role in the treatment of this disease, 80% of the initial responders fail subsequent therapy due to the development of resistance to platinum and other chemotherapeutic agents (Beesley et al. 1997). Therefore, overall survival of the patients over the past three decades has not improved despite the introduction of new agents. For instance, taxol provides a 15-20% response rate in resistant disease, but the median survival is ~ 8 months in responders (Gore et al. 1995), which does not change the already low 5-year survival rate.

Resistance to cisplatin is mutifactorial, which includes reduced drug accumulation, and increased intracellular GSH, GSH-S-tranferase, metallothionein, DNA damage-tolerance and repair of DNA adducts (Kelly & Rozencweig 1989; Andrews & Howell 1990; Fink et al. 1996; Johnson et al. 1997; Eastman & Schulte 1998). These factors can prevent the induction of apoptosis normally seen in cells following drug exposure (Kastan et al. 1991; Fritsche et al. 1993; Hartwell & Kastan 1994). Another factor that is implicated in cisplatin resistance is the p53 tumor suppressor (Siddik et al. 1999). The p53 protein is a transcription factor that is regulated by various types of cellular stress (Giaccia & Kastan 1998; Prives 1998). In response to DNA damage, induction of p53 protein either causes cells to arrest in different phases of the cell cycle by regulating various cyclins and cyclin-dependent kinases or if damage is excessive, p53 leads the cells through apoptosis by regulating the bcl-2 gene family (Weinberg 1991; Kuerbitz et al. 1992; Lane 1992).

Although resistance to cisplatin may arise through mutation in the p53 gene (Kigawa et al. 2001), loss of p53 induction and function can also occur in the wild-type state (Siddik et al. 1999).

Following DNA damage, various functions of p53 protein are regulated by a series of post-translational phosphorylation and acetylation events (Sionov & Haupt 1999). The initial response to DNA damage is stabilization of p53 protein which primarily occurs due to the phosphorylation of p53 at the N-terminus and the resulting disruption of its interaction with the negative regulator Mdm2 (Shieh et al. 1997). Members of PI3-K family, such as DNA-PK (Lees-Miller et al. 1990), ATM (Banin et al. 1998; Canman et al. 1998; Khanna et al. 1998) and ATR (Tibbetts et al. 1999), are known to phophorylate p53 at Ser-15. Several studies have shown that phosphorylation at Ser-15 is a principal event that disrupts the negative interaction between p53 and Mdm2 protein (Meek 1998; Dumaz & Meek 1999; Jayaraman & Prives 1999). Another critical modification of p53 has been reported to occur through phosphorylation of Ser-392 at C-terminus by casein kinase II. Phosphorylation at this site, which is modified only by UV and not by IR, is considered to be significant as it activates p53 for DNA binding activity in cells (Kapoor & Lozano 1998; Kapoor et al. 2000). Kapoor et al (2000) have further demonstrated that phosphorylation at multiple sites (such as Ser-15 and Ser-392) is required for efficient transactivation function of p53 following DNA damage with UV radiation. Although there are no direct reports of alterations in phosphorylation status as a result of drug resistance, several studies have demonstrated that absence of ATM kinase activity in *ataxia telangiectasia* suppresses Ser-15 phosphorylation and disrupts p53 function (Meyn, 1995; Banin et al. 1998 ; Lee & McKinnon, 2000).

Our laboratory has been interested in designing and developing novel platinum agents in order to circumvent cisplatin resistance, and this has resulted in our identifying DACH-Ac-Pt [(1R,2R-diaminocyclohexane)(trans-diacetato)(dichloro)platinum^{IV}] as an interesting lead (Siddik et al. 1999). Previous studies have demonstrated that, in contrast to X-rays, CDDP was unable to induce significant levels of p53 and subsequent transactivation of p21$^{WAF1/CIP1}$ in 2780CP (CDDP-resistant) human ovarian cancer cells (Siddik et al. 1998). Furthermore, DACH-Ac-Pt exhibits superior activity against CDDP-resistant human ovarian cancer cells harboring wild-type p53 (Hagopian et al. 1999, Siddik et al. 1999). We have postulated that the normal p53 pathway activated by CDDP is disrupted in resistant cell lines, and that DACH-Ac-Pt uses an alternative pathway to affect p53 induction and function. This study was conducted to support this postulate by understanding the differential effects of CDDP and DACH-Ac-Pt on post-translational modifications of p53 at the critical Ser-15 and Ser-392 sites, and studying p53 function through the downstream effectors p21$^{WAF1/CIP1}$ and Mdm-2 in CDDP-sensitive (A2780 and MCF-7) and CDDP-resistant (2780CP/Cl-16 and HEY/C-2) cell lines. In addition, we have also evaluated the role of PI3-K in modulation of p53 phosphorylation, and onset of p53-dependent downstream events in response to both CDDP and DACH-Ac-Pt.

Materials and Methods

Chemicals:

Cisplatin was synthesized as described previously (Vollano et al. 1987). The synthesis and chemical characterization of DACH-Ac-Pt has also been reported previously

(Siddik et al. 1999). Cisplatin and DACH-Ac-Pt were dissolved in normal saline and water, respectively, and sterilized by fitering through 0.22 μm disc filters. The concentration of each drug was determined by its platinum metal content by flameless atomic absorption spectroscopy (Siddik et al. 1987). MTT and propidium iodide were purchased from Sigma Chemical Company (St. Louis, MO) and fetal calf serum was purchased from Atlanta Biological (Norcross, GA).

Cell lines and Antibodies:

The A2780 line was established from a patient's biopsy prior to initiation of any chemotherapeutic regimen (Godwin et al. 1992). 2780CP/Cl-16 (used in this study) was selected as a single clone from the A2780CP cell line, which was previously established in this laboratory from cisplatin-resistant A2780/C30 cells (Siddik et al. 1998). The HEY cell line (Hamaguchi et al. 1993) was established from a patient who had failed cisplatin therapy and is classified as cisplatin-resistant (Buick et al. 1985). The HEY/C-2 model (used in this study) is a clone derived from parental HEY cells, and was a kind gift from Dr. Gordon Mills of M.D. Anderson Cancer Center. The MCF-7 breast cancer cell line was obtained from American Type Tissue Culture Collection (Manassas, VA). Cells were grown in RPMI containing 10% fetal calf serum, 1mM glutamine and antibiotics (100 μg/ml Streptomycin and 100 U/ml Penicillin). All the cell lines used in this study have wild-type p53 genotype and/or function. Human specific p53 (DO-1) and Mdm-2 (SMP 14) antibodies were obtained from Oncogene Research Products (Cambridge, MA) and Santa Cruz (Santa Cruz, CA), respectively. Phospho-specific p53 antibodies (Ser-15: 9284S; Ser-392: 9281S) were purchased from New England Biolabs (Beverly, MA). Monoclonal $p21^{WAF1/CIP1}$ antibody

(C24420) was obtained from Transduction Laboratories (Laxington, KY) and monoclonal β-actin antibody (AC-15) was obtained from Sigma Chemical Co. (St Louis, MO).

Cytotoxicity Studies:

The effect of CDDP and DACH-Ac-Pt on growth of A2780, 2780CP/Cl-16, MCF-7 and HEY/C-2 cells was determined by a modified MTT assay using the continuous drug treatment (3-5 days) protocol reported previously from our laboratory (Siddik et al. 1998; Hagopian et al. 1999). Briefly, human cancer cells (500-1500 cells) were plated into 96-well plates in 100 μl aliquots and allowed to adhere for 24 h at 37°C. The stock solutions of CDDP and DACH-Ac-Pt were diluted in complete media immediately before use and added to the wells in quadruplicate at various concentrations in 100 μl volume. After 3-5 days at 37°C, 50 μl-aliquots of an MTT solution (3 mg/ml) were added to each well and plates were incubated for an additional 4 h at 37 °C. The media was then removed and replaced with 50 μl of 100% DMSO to dissolve the formazan crystals with agitation for 5-10 mins on a shaker. The absorbance was measured at 570 nm using a multiwell scanning spectrophotometer (Molecular Devices, Sunnyvale, CA). The IC_{50} values were determined from a plot of log concentration against A_{570} readings as a percentage of control.

Western Immunoblot Analysis:

Although the cytotoxicity study employed a continuous drug exposure protocol, Western analysis were conducted following a 2-h exposure using a correspondingly higher drug concentration. This was necessary to synchronize induction of p53 and transactivation of its downstream gene targets. It is important to note, however, that the concentrations used here are similar to or lower than the IC_{50} of 30 and 112 μM in HEY and 2780CP models, respectively, using the 2-h drug exposure protocol (Siddik et al. 1998; Hagopian et al. 1999). Although the highest concentration of 40 μM induced 80-90% cell kill in A2780 and MCF-7 cells, sufficient number of cells remained to successfully permit Western blot analysis.

For the analysis, ovarian and breast cancer cells were exposed to cisplatin or DACH-Ac-Pt (5-40 μM) for 2h, washed and further incubated in drug-free-media for [redacted] h at 37°C. The cells were then washed with PBS and lysed in lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate) containing 1 mM sodium vanadate, 100 μg/ml PMSF and 10 μg/ml of aprotinin for 30 mins on ice. Cell lysates were centrifuged at 14,000 rpm for 10 min. and their protein content estimated by the Lowry procedure. Equal protein aliquots of cell lysates were resolved on SDS-PAGE and transferred to nitrocellulose membranes in a semi-dry transfer unit for 2 h at room temp. The membranes were blocked for 2h in blocking buffer (5% BSA in TBS) and incubated with specific antibodies overnight at 4°C or for 15 min to 1 h at room temperature. Proteins were detected with HRP-conjugated secondary antibodies (Goat anti-rabbit or goat anti-mouse horseradish peroxidase conjugate) and visualized using the enhanced chemiluminescence assay (Amersham Biosciences, England). Quantitation of bands by laser densitometry allowed assessment of increases in proteins compared to controls.

Results

Cytotoxic effects of CDDP and DACH-Ac-Pt against CDDP-sensitive and -resistant human cancer cells:

The cytotoxicities of CDDP and DACH-Ac-Pt against human ovarian A2780, 2780CP/Cl-16 and HEY/C-2 and breast MCF-7 cell lines are shown in Table 1. A2780 and MCF-7 cells exhibited an IC_{50} of 0.31 and 0.36 μM for CDDP and 0.089 and 0.20 μM for DACH-Ac-Pt using a continuous drug exposure protocol for 3-5 days. As anticipated, HEY/C-2 and 2780CP/Cl-16 cells exhibited resistance to CDDP with an IC_{50} of 3.9 and 7.1 μM, respectively, and sensitivity to DACH-Ac-Pt with a corresponding IC_{50} of 0.60 and 0.29 μM. These results indicate a differential cellular expression of fold-resistance to the two drugs, and demonstrate that DACH-Ac-Pt is able to circumvent cisplatin resistance in HEY/C-2 and 2780CP/Cl-16 cells (Table 1). This is expected based on our previous investigations with CDDP-resistant cell lines possessing wild type p53 function (Hagopian et al. 1999; Siddik et al. 1999).

Induction and phosphorylation of p53:

To investigate the effect of CDDP and DACH-Ac-Pt on p53 induction and Ser-15 phosphorylation, we conducted a time-course study in tumor cells to establish temporal profiles for increases in total and phosphorylated p53 levels. A2780 and 2780CP/Cl-16 cells were exposed to 35 μM CDDP or DACH-Ac-Pt for 2h, washed with PBS, incubated in drug-free-media, and harvested at different time-points up to 72 hours. Cell lysates were analyzed by Western-blotting and β-actin was used to demonstrate equivalent protein loading on the gels. As shown in Figure 1A, a time-dependent induction of total and phosphorylated p53 protein was observed in A2780 cells in response to both CDDP and DACH-Ac-Pt. The induction peaked at 12-48 hours with CDDP, whereas maximum p53 induction was observed at 6-24 hours with DACH-Ac-Pt, demonstrating that DACH-Ac-Pt induced p53 more rapidly. From a temporal perspective, induction of total and phosphorylated p53 in resistant 2780CP/Cl-16 cells (Fig. 1B) paralleled in general those observed in sensitive cells.

It is noteworthy that the induced levels of p53 in 2780CP/Cl-16 cells were lower compared to A2780 cells, and this was consistent with the relative sensitivities of the two tumor models to the platinum agents. To strengthen these observations, the studies were extended to the HEY/C-2 resistant cell line, but using a dose-response approach and a 24h time-point as an optimal period to assess induction. HEY/C-2 cells, together with sensitive A2780 and MCF-7 cell lines, were exposed to 5-40 μM of CDDP or DACH-Ac-Pt for 2h, followed by incubation in drug-free media for 24h and the immunoblots were prepared as before. The results in Figure 2 demonstrate a dose-dependent induction of total p53 protein in response to both CDDP and DACH-Ac-Pt in the three cell lines. Compared to controls, we observed a 4-9 fold increase in levels of p53 protein following exposure to both CDDP and DACH-Ac-Pt at the highest concentration in CDDP-sensitive A2780 and MCF-7 cells. In contrast, a relatively lower 2-4 fold induction of total p53 protein was observed in resistant HEY/C-2 cells, and this is consistent with the reduced level of induction seen in resistant 2780CP/Cl-16 cells (Fig. 1).

Dose-dependent increases in p53 phosphorylation at Ser-15 in response to CDDP were seen in A2780, MCF-7 and HEY/C-2 cells (Fig. 2A-C). Increases in Ser-15 phosphorylation were 7-15 fold in A2780, 5-15 fold in MCF-7 and 2-8 fold in HEY/C-2 cells in response to CDDP across the range of concentrations used. Interestingly, the increase in Ser-15 phosphorylation was 2-3 fold greater in CDDP-sensitive compared to CDDP-resistant HEY/C-2 cells, and this again is consistent with the observation with 2780CP/Cl-16 cells (Fig.1). DACH-Ac-Pt also showed an increase in p53 phosphorylation at Ser-15; however, the increases were less pronounced (2-6 fold in A2780, 2-4 fold in MCF-7 and 2-3 fold in HEY/C-2 cells) compared to CDDP.

Increase in p53 phosphorylation at Ser-392 was also investigated as a dose-response relationship in A2780, HEY/C-2 and MCF-7 cells (Fig. 2). CDDP induced Ser-392 phosphorylation in a dose-dependent manner in all the three cell lines. In contrast, phosphorylation at Ser-392 with DACH-Ac-Pt was not induced to any appreciable extent in MCF-7 (Fig. 2B) and HEY/C-2 cells (Fig. 2C), and only a slight increase in phosphorylation at this site was detected in A2780 cells (Fig. 2A). These results clearly demonstrate that CDDP and DACH-Ac-Pt activate independent DNA-damage signaling pathways.

Transactivation function of p53:

Induction of p53 and its functional activation are separable events, and it was necessary to assess whether the differential effects of the platinum drugs on p53 levels were consistent with observations of p53 function. This function may be monitored by following transcriptional activation of $p21^{WAF1/CIP1}$ and Mdm2 as important downstream effectors of the p53 pathway. We first examined the effect of CDDP and DACH-Ac-Pt on increases in $p21^{WAF1/CIP1}$ protein in a time course study. Figure 3A demonstrates that the increases in $p21^{WAF1/CIP1}$ were observed in A2780 cells from 6h onwards and reached maximal levels at 24-48h with CDDP and 12-24h with DACH-Ac-Pt. Interestingly, these increases lagged behind increases in p53 levels observed in Figure 1, and this is consistent with p53-mediated transactivation of p21$^{WAF1/CIP1}$. In contrast, in CDDP-resistant 2780CP/Cl-16 cells, a time-dependent induction of p21$^{WAF1/CIP1}$ protein, albeit at a relatively reduced level, was observed only in response to DACH-Ac-Pt with maximum induction observed 24-48 h after the treatment: p21$^{WAF1/CIP1}$ was not induced by CDDP in resistant 2780CP/Cl-16 cells (Fig 3A).

The dose-dependent study in A2780 and MCF-7 cells confirmed the robust induction of p21$^{WAF1/CIP1}$ protein in response to both CDDP and DACH-Ac-Pt in sensitive models (Fig. 3B). Quantitatively, a 9-23 fold induction of p21$^{WAF1/CIP1}$ in response to CDDP and 10-27 fold induction in response to DACH-Ac-Pt were observed in A2780 cells. In MCF-7 cells, we observed a 2-3 fold induction with CDDP and 2-4 fold induction with DACH-Ac-Pt (Fig. 3B). Thus, both drugs induced p21$^{WAF1/CIP1}$ to similar extents in sensitive cells. On the other hand, and as was seen with 2780CP/Cl-16 cells, induction of p21$^{WAF1/CIP1}$ protein in HEY/C-2 cells was observed only in response to DACH-Ac-Pt, but not with CDDP.

In order to determine if the differential p21$^{WAF1/CIP1}$ response in resistant cells exposed to CDDP was a specific or a generalized transactivation dysfunction of p53 in resistance, parallel studies were undertaken to examine Mdm2 as an alternative target of p53. Results in Figure 4 indicate the rapidity with which Mdm2 is induced, and that this induction was both time- and dose-dependent in sensitive and resistant cells. Moreover, the levels induced in these cells were similar with both Pt drugs (2-5 fold). These results demonstrate that, unlike p21$^{WAF1/CIP1}$, transactivation of Mdm-2 was not inhibited in resistant cells.

Role of PI3-K on phosphorylation of p53:

Several members of the phosphotidyl-inositol-3-kinase (PI3K) family phosphorylate p53 at Ser-15 as a process of p53 activation following exposure to UV and IR radiation (Lees-Miller et al. 1990; Price & Youmell 1996; Rosenzweig et al. 1997; Banin et al. 1998; Canman et al. 1998; Khanna et al. 1998; Tibbetts et al. 1999). In order to examine the role PI3-K plays in p53 induction and function in the present study, and to confirm that transactivation of p21$^{WAF1/CIP1}$ was mediated via p53 phosphorylation, we used wortmannin as a PI3-K inhibitor in our studies. Since p21$^{WAF1/CIP1}$ induction was absent in resistant cells following CDDP treatment, the two sensitive cell lines were selected for these investigations. The cells were pretreated with wortmannin (1, 3 or 10 μM) for 2h, then exposed for 2h to 20 μM CDDP or DACH-Ac-Pt in the presence of wortmannin, and treatment with wortmannin alone continued for an additional 6h (A2780 cells) or 20h (MCF-7 cells). The different total exposure time to wortmannin reflects the relative sensitivities of the two cell lines to the inhibitor. Following the final exposure period to wortmannin, the cells were harvested for western analysis. DMSO, the solvent for dissolving wortmannin, was used as a control.

In Fig. 5, the induction and phosphorylation of p53 and increase in p21$^{WAF1/CIP1}$ levels in A2780 cells are evident following platinum treatment. The low level of p21$^{WAF1/CIP1}$ observed with CDDP was consistent with the early time-point of investigation. With wortmannin, a dose–dependent inhibition of total p53 protein accumulation and Ser-15 phosphorylation was observed. However, phosphorylation was not inhibited completely even at the highest wortmannin concentration, suggesting that other kinases may be involved in phosphorylation at this site. In contrast, the PI3-K inhibitor almost completely inhibited p21$^{WAF1/CIP1}$ induction at the lowest concentration, but only partially inhibited total and phosphorylated p53.

In A2780 cells, the greater sensitivity to wortmannin precluded extension of incubation time beyond 6h following the platinum treatment, which did not allow a robust increase in p21$^{WAF1/CIP1}$ with CDDP or an unequivocal demonstration of inhibition of p21$^{WAF1/CIP1}$ induction. For this reason, and to confirm observations in a second cell line, we conducted limited inhibition studies in MCF-7 cells. Figure 6 confirms the finding seen in A2780 cells that the induced Ser-15 phosphorylation by the platinum compounds in MCF-7 cells was inhibited incompletely by wortmannin. Although inhibition of induced p21$^{WAF1/CIP1}$ levels by wortmannin was apparent, the basal levels of p21$^{WAF1/CIP1}$ were not affected by the inhibitor. Thus, wortmannin inhibited only the increased levels of p21$^{WAF1/CIP1}$, which presumably occurred as a result of p53 activation. Wortmannin did not cause any changes in Ser-392 phosphorylation in these cells (data not shown). These results demonstrate that members of PI3-K family are involved in phosphorylating and functionally activating p53 in response to DNA damage by both CDDP and DACH-Ac-Pt.

Discussion

We have demonstrated that although DACH-Ac-Pt was 2- to 3-fold more cytotoxic than CDDP against cisplatin-sensitive cells, the differential cytotoxicity favoring the analog was even greater (10-20 fold) against CDDP-resistant 2780CP/Cl-16 and HEY/C-2 cells. This is consistent with our previous findings regarding the ability of DACH-Ac-Pt to circumvent CDDP resistance in tumor models harboring wild-type p53 tumor suppressor protein and with our proposal that the two agents activate p53 through independent signaling pathways (Siddik et al., 1998; Hagopian et al. 1999). In the present study, we sought to investigate whether the postulated independent pathways regulate p53 by post-translational modification at distinct or identical sites. Our findings indicate that there are similarities and differences in p53 phosphorylation at Ser-15 and Ser-392 sites following exposure of tumor cells to the two drugs.

Numerous reports in the literature have shaped our understanding that DNA damage activates multiple signal transduction pathways, which regulate the biological function of p53 tumor suppressor protein by modulating phosphorylation and acetylation status at numerous sites (Appella & Anderson 2001). Post-translational modification of Ser-15 is demonstrated with a variety of DNA damaging agents, and our present observation that exposure to either cisplatin or DACH-Ac-Pt results in phosphorylation of this site is in keeping with the reported effects of DNA damage. Phosphorylation at Ser-15 is an important step in stabilizing p53 by disrupting its interaction with the negative regulator Mdm-2, which otherwise targets p53 for ubiquitin-mediated degradation (Vousden 2002). This disruption also occurs with both platinum drugs, as these agents induced parallel increases in Ser-15-phosphorylated and total p53 in sensitive tumor cells. The parallel increases in total and the phosphorylated form of p53 were also seen with the analog in resistant cells, but it is interesting that exposure of these cells to cisplatin did not induce high levels of total p53 that were consistent with increases in Ser-15 phosphorylation. This may be related to reduced Ser-15 phosphorylation with cisplatin in resistant cells compared to robust increases observed in sensitive cells, but this explanation is weakened by the observation that similar or lower level of p53 phosphorylation were also observed with

DACH-Ac-Pt in both sensitive and resistant models. Thus, translation of Ser-15 phosphorylation into total p53 induction occurs with greater efficiency when the analog is the DNA damaging agent. A possible explanation for this is that even though Ser-15 phosphorylation occurs with both cisplatin and DACH-Ac-Pt, the phosphorylation of p53 is not qualitatively identical, perhaps due to differential protein phosphorylation at additional site(s).

An additional critical p53 phosphorylation site is Ser-392, which facilitates DNA binding function (Kapoor & Lozano 1998; Kapoor et al. 2000). The present studies demonstrate that this site is also phosphorylated following DNA damage by cisplatin, but DACH-Ac-Pt induced little or no increase in levels of Ser-392-p53. This differential phosphorylation of Ser-392 by the two platinum agents is a highly significant observation for drugs of the same class, and strongly supports our premise that independent signal transduction pathways are involved in mediating the DNA damage signals induced by cisplatin and the analog. It is interesting to note that these observations mirror those reported for UV and IR; more specifically, phosphorylation at Ser-392 in human tumor cells (or the equivalent Ser-389 in murine cells) is induced by UV but not by IR, suggesting that different DNA damaging agents activate p53 through distinct mechanisms (Kapoor & Lozano 1998; Lu et al. 1998; Kapoor et al. 2000). Phosphorylation at Ser-392 by UV and cisplatin provides an important close signaling link between the two agents, and suggests activation of a common mechanism. However, Ser-392 is known to be phosphorylated by casein kinase II (Laskin & Jackson 1999), and there is no information to directly connect cisplatin and UV to this kinase. On the other hand, cisplatin and UV can both activate the ATR kinase, which is known to phosphorylate p53 at Ser-15 (Tibbetts et al. 1999; Damia et al. 2001; Zhao and Piwnica-Worms, 2001), but the possibility of regulation of casein kinase II by ATR is not known at the present time. In an analogous comparison, the lack of phosphorylation at Ser-392 by IR and DACH-Ac-Pt is also suggestive of invoking a common mechanism by these two agents. This is bolstered by the finding that both agents can circumvent cisplatin resistance (Siddik et al., 1998; present study). Since the ATM kinase, which also phosphorylates p53, is known to be activated in response to IR (Banin et al 1998; Canman et al 1998; Hirao et al. 2002), it is possible that DACH-Ac-Pt may also mediate its effects through the ATM pathway. It is noteworthy that both ATM and ATR belong to the PI3-K family, and, therefore the inhibition of p53 phosphorylation and induction and p21$^{WAF1/CIP1}$ transactivation by the PI3-K inhibitor wortmannin provides strong support for the involvement of these or similar kinases in the mechanism of p53 modification by cisplatin and DACH-Ac-Pt.

A major outcome of p53 phosphorylation is to enable the tumor suppressor to transctivate a number of genes that are essential for cell cycle arrest and apoptosis (Laskin & Jackson 1999; Sionov & Haupt 1999). We have shown that p53 induction led to transactivation of two downstream effectors, p21$^{WAF1/CIP1}$ and Mdm-2, in CDDP-sensitive tumor cells in response to both CDDP and DACH-Ac-Pt. However, induction of p21$^{WAF1/CIP1}$ protein was observed only in response to DACH-Ac-Pt in CDDP-resistant tumor models, which is consistent with our previous observations (Hagopian et al. 1999). The lack of p21$^{WAF1/CIP1}$ induction by cisplatin in resistant cells, even though phosphorylations at Ser-15 and Ser-392 were observed, is an important finding and indicates the presence of a significant inhibitory step that prevents the p53 protein from acquiring full transactivation capacity. In contrast, Mdm-2 induction was observed with both Pt drugs in the resistant models, suggesting that the inability of cisplatin-induced p53 to transactivate $p21^{WAF1/CIP1}$ in resistant cells is restricted to this specific gene. In this regard, it is useful to indicate that in a previous report, CDDP-mediated induction of $p21^{WAF1/CIP1}$ was shown to be critically regulated by the PI3-K/AKT pathway (Mitsuuchi et al. 2000), and this is not inconsistent with our finding that wortmannin inhibited increases in levels of $p21^{WAF1/CIP1}$ through inhibition of PI3-K. As suggested above, the differential $p21^{WAF1/CIP1}$ transactivation, but not that of Mdm2, following cisplatin and DACH-Ac-Pt exposure points to inhibition of a significant activation step for p53 when cisplatin is the mediator of DNA damage. Whether this inhibitory step is related to incomplete cisplatin-mediated post-translational modification of p53 is not known, but is possible and could provide a plausible explanation for the selective loss of $p21^{WAF1/CIP1}$ transactivation.

A major outcome from our studies is that the status of p53 phosphorylation at Ser-392 is not essential for transactivation of $p21^{WAF1/CIP1}$ or Mdm-2, nor is it related to cisplatin resistance. Whether an absence of Ser-392 phosphorylation enables p53 induced by DACH-Ac-Pt to facilitate apoptosis in cisplatin-resistant cells cannot be excluded. Our findings do raise the notion that induction of $p21^{WAF1/CIP1}$ by DACH-Ac-Pt in resistant models may have a role in circumvention of CDDP-resistance. This, however, goes against the literature understanding that an increase in $p21^{WAF1/CIP1}$ is generally associated with an increased level of resistance to some chemotherapeutic drugs (Waldman et al. 1996; Fan et al. 1995; Fan et al. 1997). Nevertheless, ectopic expression of $p21^{WAF1/CIP1}$ in human ovarian carcinoma cells has been shown to reduce tumor cell growth and enhance susceptibilty to cisplatin-induced apoptosis (Lincet et al. 2000). Similarly, $p21^{WAF1/CIP1}$ induction has been associated with cell death in cervical carcinoma cells (Tsao et al. 1999). Thus, the involvement of p21$^{WAF1/CIP1}$ in the cytotoxicity of DACH-Ac-Pt cannot be totally ruled out.

In conclusion, our studies have clearly shown that CDDP and DACH-Ac-Pt differentially phosphorylate p53 at Ser-392, which interestingly does not affect p21$^{WAF1/CIP1}$ or Mdm-2 transactivation. However, cisplatin resistance and its circumvention by DACH-Ac-Pt appears to correlate directly with regulation of p21$^{WAF1/CIP1}$ levels by these agents. Further studies are needed to examine this correlation closely and the contribution to this of post-translational modification of p53 with the two platinum agents.

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Figure legends:

Figure 1. Temporal induction of total p53 protein and phosphorylation of p53 at Ser-15 in response to Pt drugs in CDDP-sensitive and -resistant human ovarian cells: A2780 (CDDP-sensitive; A) and 2780CP/Cl-16 (CDDP-resistant; B) cells in exponential growth were exposed to CDDP and DACH-Ac-Pt (35 μM) for 2h, washed and then incubated in drug-free medium and harvested at the indicated times. The proteins were examined for p53 levels and its phosphorylation at Ser-15 by western blotting. Con, control.

Figure 2. Dose-response of CDDP and DACH-Ac-Pt on p53 induction and activation of p53 phosphorylation: The A2780 (CDDP-sensitive; 2A), MCF-7 (CDDP-sensitive; 2B) and HEY/C-2 (CDDP-resistant; 2C) cells were exposed to different concentrations (5-40 μM) of Pt drugs for 2h, washed and incubated in drug-free media for 24 h at 37°C. The protein was extracted and examined by Western-ECL technique using anti-p53 (total) and phosho-specific (Ser-15 and Ser-392) antibodies as probes. Con, control.

Figure 3: Induction of $p21^{WAF1/CIP1}$ in response to Pt drugs in CDDP-sensitive and –resistant cancer cells (time-course and dose-response study): A2780 and 2780CP/Cl-16 cells (3A) were treated with 35 μM of either CDDP or DACH-Ac-Pt for 2h, washed, incubated in drug-free medium and harvested at indicated time points. In Figure 3B, A2780, MCF-7 and HEY/C-2 cells were treated with varying concentrations of Pt drugs (5-40 μM) for 2h followed by 24h incubation in drug-free medium. $p21^{WAF1/CIP1}$ was detected by Western blotting. Con, control.

Figure 4: Induction of Mdm-2 in CDDP-sensitive and -resistant human ovarian cancer cells: The protein from 2780CP/Cl-16 cells (exposed to 35 μM Pt drug for 2h) (4A) or A2780 and HEY/C-2 cells (2h drug exposure, then 24h incubation in drug free medium) (4B) was extracted and examined for Mdm-2 protein by western-ECL analyses using anti-Mdm-2 antibody as a probe. β-actin was used to confirm equal protein loading. Con, control.

Figure 5: Modulation of p53 and p21$^{WAF1/CIP1}$ levels by PI3-K inhibitor wortmannin: A2780 cells were pretreated (2h) with different concentrations (1, 3 and 10 μM) of wortmannin alone, followed by a further 2h exposure in the presence of CDDP or DACH-Ac-Pt. The cells were then washed and exposed to wortmannin for an additional 6h. The proteins were extracted and detected with respective antibodies using Western blotting.

Figure 6: Dose-dependent regulation of p53 and p21 $^{WAF1/CIP1}$ by wortmannin in MCF-7 cells: MCF-7 cells were pretreated with wortmannin alone for 2h, followed by a further 2h treatment in the presence of CDDP or DACH-Ac-Pt. After washing, the cells were further exposed to wortmannin for 20 h. Protein was extracted and detected with specific antibodies to p53, Ser-15 and p21. β-actin was used as a loading control. Con, control.

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Table 1

IC$_{50}$ of Cisplatin and DACH-acetato-Pt on CDDP-sensitive and -resistant cancer cells

		IC$_{50}$ (µM)		Fold resistance relative to A2780	
Cell line	Cell type	Cisplatin	DACH-acetato-Pt	Cisplatin	DACH-acetato-Pt
A2780	ovarian	0.31 ±.034	0.089 ±.029	1	1
MCF-7	breast	0.36 ± 0.03	0.20 ± 0.07	1.2	2.3
HEY/C-2	ovarian	3.9 ± 1.0	0.60 ± 0.19	12.7	6.7
2780CP/Cl-16	ovarian	7.1 ± 0.51	0.29 ± 0.05	23	3.2

IC$_{50}$ values were determined by an MTT assay 3-5 days after exposure to a range of drug concentrations.
The 50% cell kill were determined from the log-linear plot using a sigmoidal curve function.
Mean ± SE of 3-7 independent experiments.

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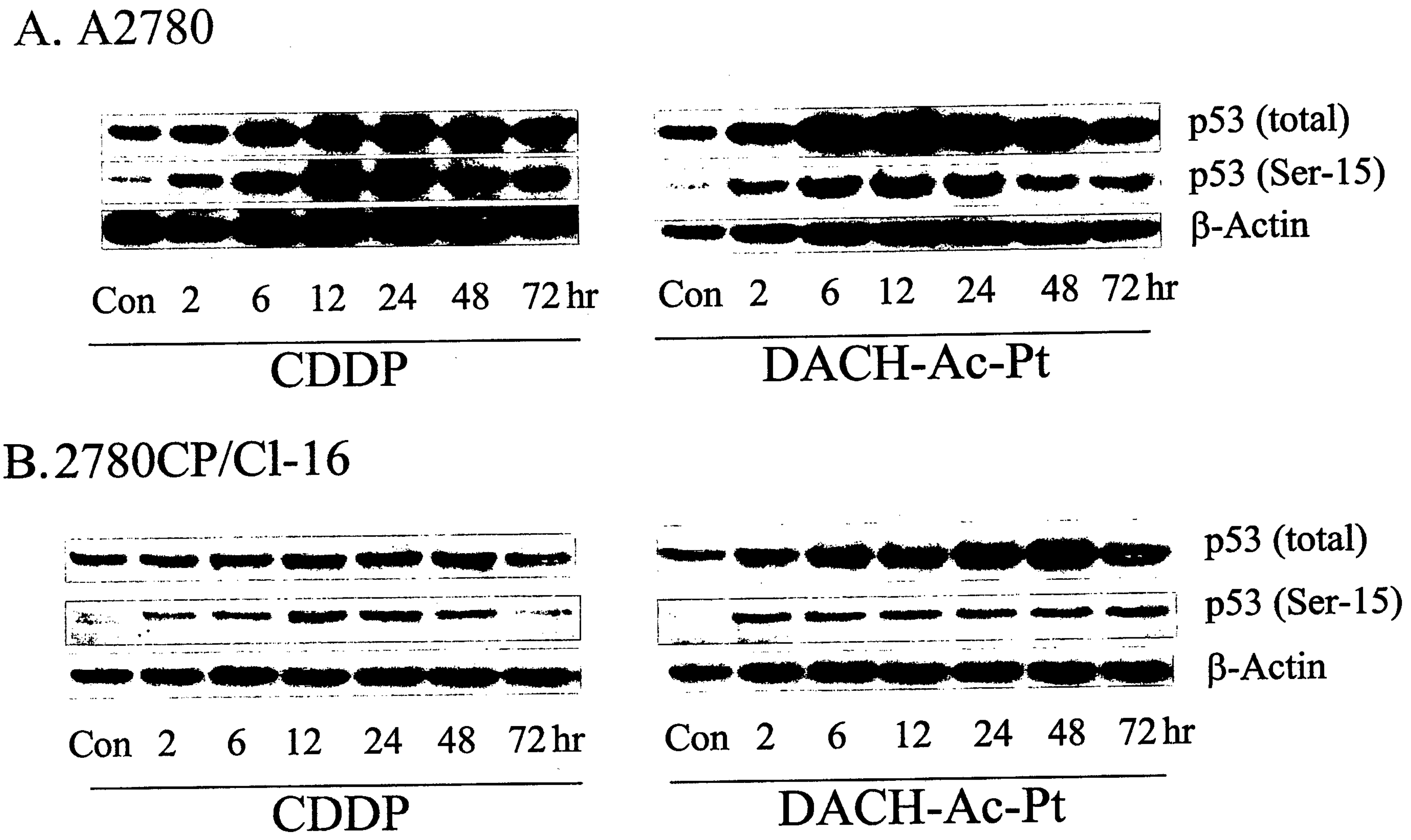
A. A2780
p53 (total)
p53 (Ser-15)
β-Actin
Con 2 6 12 24 48 72 hr
CDDP
Con 2 6 12 24 48 72 hr
DACH-Ac-Pt
B.2780CP/Cl-16
p53 (total)
p53 (Ser-15)
β-Actin
Con 2 6 12 24 48 72 hr
CDDP
Con 2 6 12 24 48 72 hr
DACH-Ac-Pt

Fig.1/Mujoo et al.

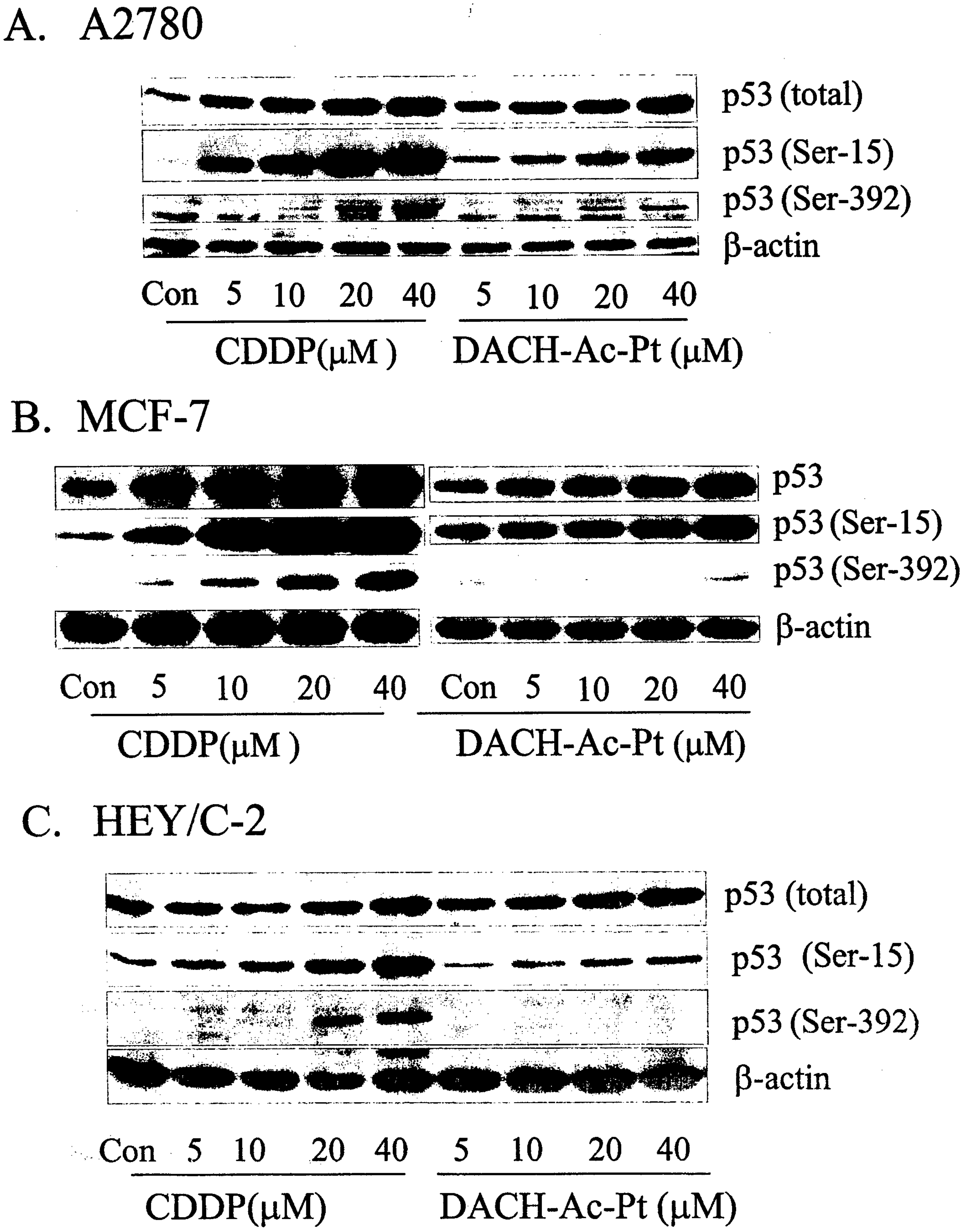
A. A2780
p53 (total)
p53 (Ser-15)
p53 (Ser-392)
β-actin
Con 5 10 20 40 5 10 20 40
CDDP(μM)
DACH-Ac-Pt (μM)
B. MCF-7
p53
p53 (Ser-15)
p53 (Ser-392)
β-actin
Con 5 10 20 40 Con 5 10 20 40
CDDP(μM)
DACH-Ac-Pt (μM)
C. HEY/C-2
p53 (total)
p53 (Ser-15)
p53 (Ser-392)
β-actin
Con 5 10 20 40 5 10 20 40
CDDP(μM)
DACH-Ac-Pt (μM)

Fig.2/Mujoo et.al.

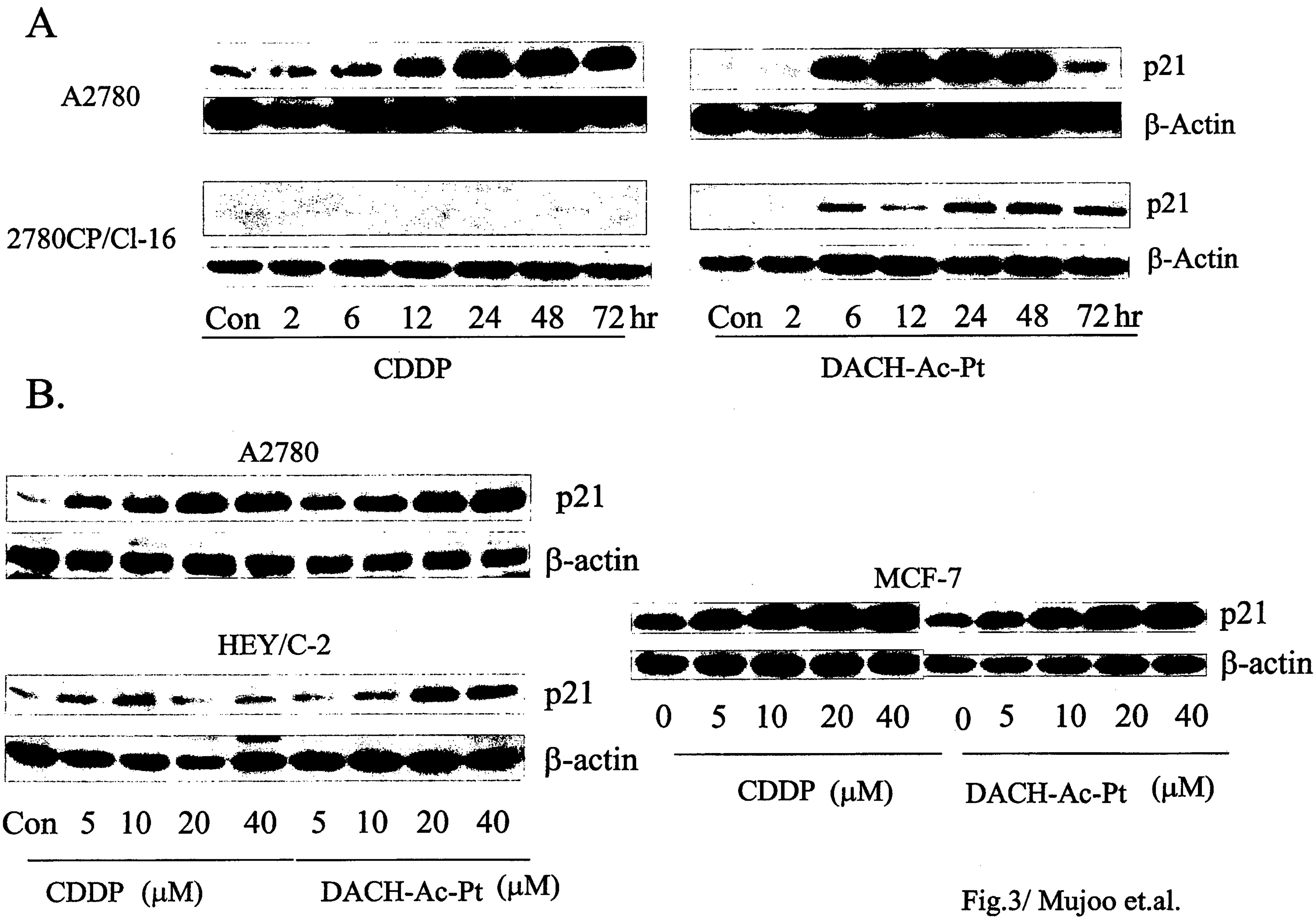
A
A2780
2780CP/Cl-16
p21
β-Actin
p21
β-Actin
Con 2 6 12 24 48 72 hr
CDDP
Con 2 6 12 24 48 72 hr
DACH-Ac-Pt
B.
A2780
p21
β-actin
HEY/C-2
p21
β-actin
Con 5 10 20 40 5 10 20 40
CDDP (μM)
DACH-Ac-Pt (μM)
MCF-7
p21
β-actin
0 5 10 20 40 0 5 10 20 40
CDDP (μM)
DACH-Ac-Pt (μM)

Fig.3/ Mujoo et.al.

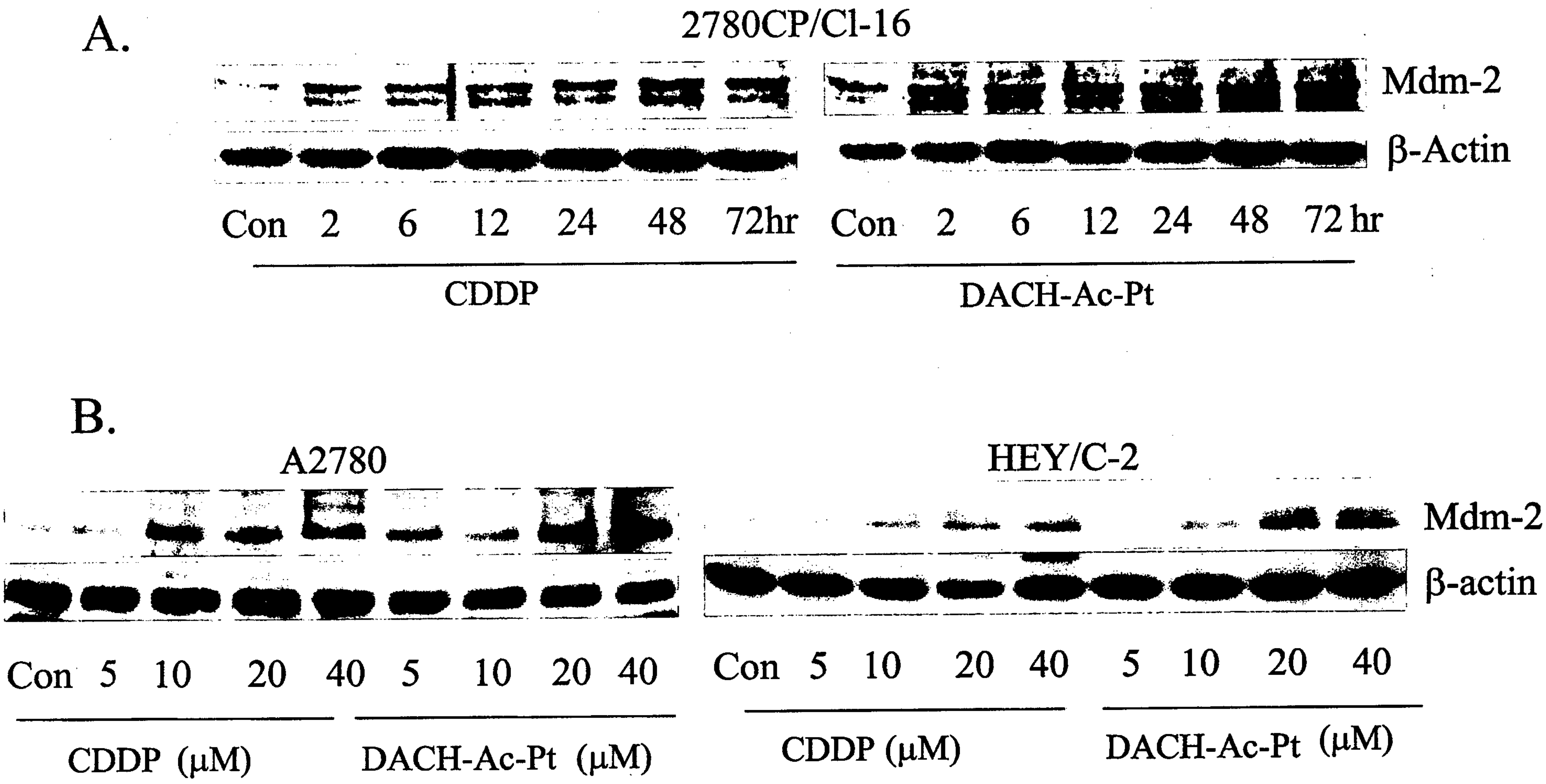
A.
2780CP/Cl-16
Mdm-2
β-Actin
Con 2 6 12 24 48 72hr
CDDP
Con 2 6 12 24 48 72 hr
DACH-Ac-Pt
B.
A2780
Con 5 10 20 40
CDDP (μM)
5 10 20 40
DACH-Ac-Pt (μM)
HEY/C-2
Con 5 10 20 40
CDDP (μM)
5 10 20 40
DACH-Ac-Pt (μM)
Mdm-2
β-actin

Fig.4/Mujoo et.al

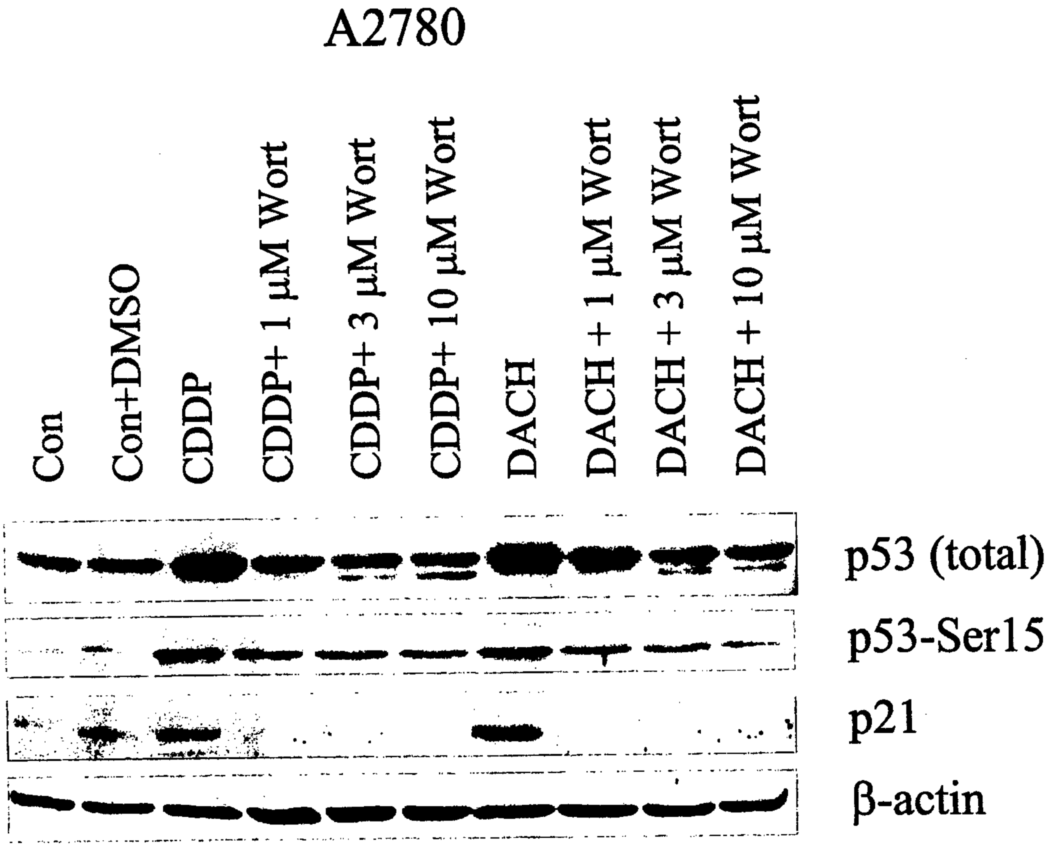
A2780
Con
Con+DMSO
CDDP
CDDP+ 1 μM Wort
CDDP+ 3 μM Wort
CDDP+ 10 μM Wort
DACH
DACH + 1 μM Wort
DACH + 3 μM Wort
DACH + 10 μM Wort
p53 (total)
p53-Ser15
p21
β-actin

Fig.5/Mujoo et.al.

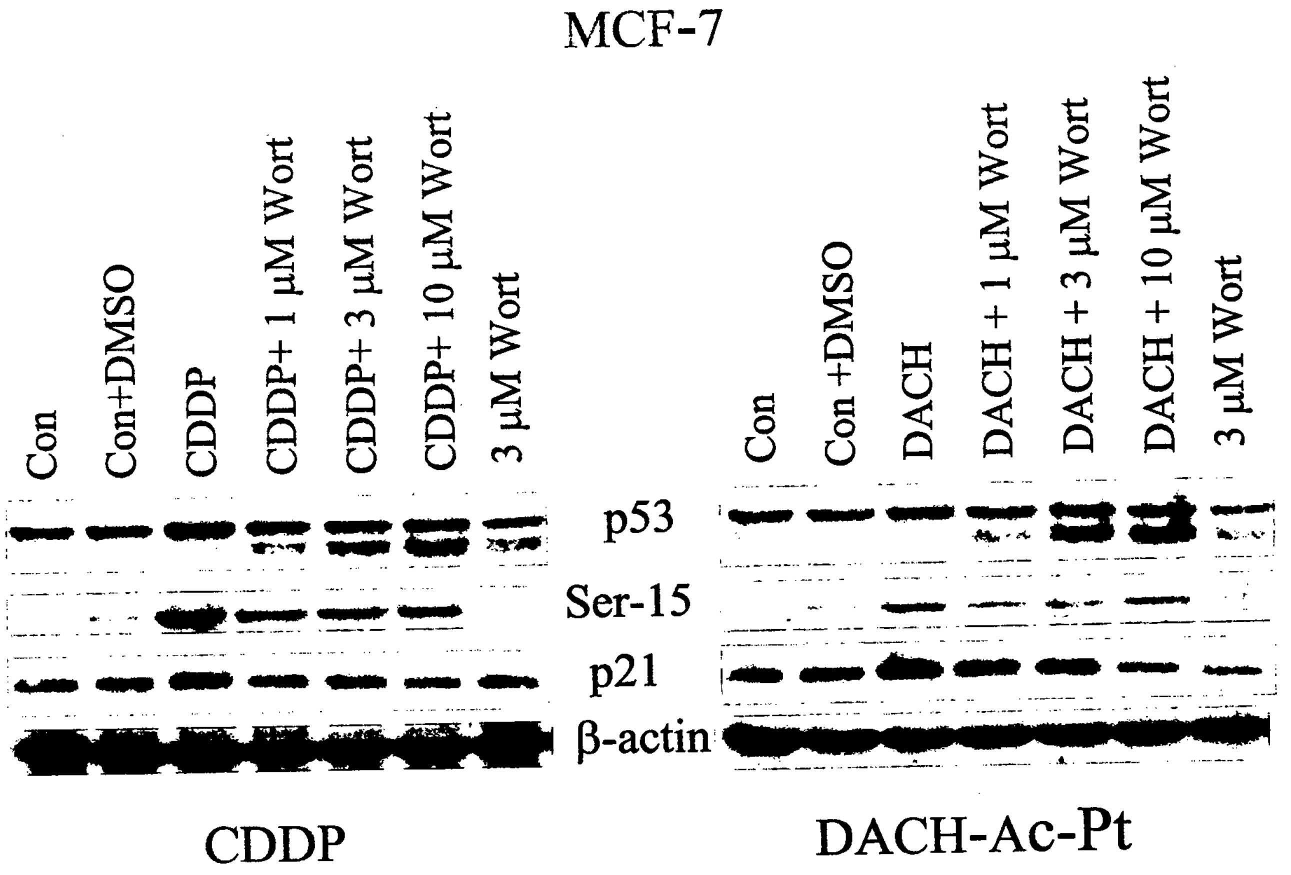
MCF-7
Con
Con+DMSO
CDDP
CDDP+ 1 µM Wort
CDDP+ 3 µM Wort
CDDP+ 10 µM Wort
3 µM Wort
Con
Con +DMSO
DACH
DACH + 1 µM Wort
DACH + 3 µM Wort
DACH + 10 µM Wort
3 µM Wort
p53
Ser-15
p21
β-actin
CDDP
DACH-Ac-Pt

Fig.6/Mujoo et.al.

Differential Modulation by HER2/neu of the Cytotoxicity of Cisplatin and 1R,2R-Diaminocyclohexane-diacetato-dichloro-Platinum (IV) Against Wild-Type and mutant p53 Breast Tumor Cells[1]

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Running Title: Modulation of platinum sensitivity by HER2 and p53

Keywords: HER2/neu, p53, platinum

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ABSTRACT

To clarify the effects of HER2/neu overexpression on the sensitivity to cisplatin and the novel analog DACH-acetato-Pt against tumor cells with different p53 status, we have utilized two stable HER2/neu transfection models bearing wild-type or mutant p53. Increased levels of HER2/neu led to an increase in resistance to cisplatin in wild-type p53 MCF-7 cells, but did not affect cisplatin cytotoxicity in mutant p53 MDA-MB-435 cells. On the other hand, HER2/neu overexpression significantly increased sensitivity to DACH-acetato-Pt independent of p53 status, although DACH-acetato-Pt was much more potent against wild-type p53 cells. Biochemical pharmacology demonstrated that the change in cytotoxicity induced by HER2/neu was due entirely to an inverse change in DNA damage tolerance. In MCF-7 cells, both drugs induced p53 in a dose- and time-dependent manner. Overexpression of HER2/neu attenuated both the induction of total p53 and its phosphorylation at serine 15 and 392 in response to cisplatin, but did not reduce p53 induction following exposure to DACH-acetato-Pt. These results suggest that independent pathways are involved in p53 activation for the two platinum agents and HER2/neu only impinges on the pathway activated by cisplatin. DACH-acetato-Pt may have utility in the management of HER2/neu-overexpressing tumors, particularly against a wild-type p53 background.

INTRODUCTION

Two of the major barriers against successful therapy of human cancers are mutation of the p53 tumor suppressor gene and an amplification/overexpression of HER2/neu gene. This is particularly relevant to several tumor types, including ovarian and breast cancers. In invasive breast cancer, for instance, the frequency of mutations in p53 gene ranges from 12-46 %, while HER2/neu is overexpressed in 10 to 34 % (Berns et al. 2000; Ross and Fletcher 1998). Both of these genetic alterations are reported to be powerful predictors not only of survival but also of tumor response to adjuvant therapy (Andersen and Borresen 1995; Burke et al. 1998).

Wild-type p53 is a DNA binding protein, which acts as a transcriptional factor to control the expression of a variety of genes regulating growth arrest and apoptosis (Albrechtsen et al. 1999). In addition to transactivating the cyclin-dependent kinase inhibitor $p21^{waf1/Cip1}$ (El-Deiry 1997), p53 also upregulates MDM2, which binds p53 and acts as an ubiquitin ligase (Honda et al. 1997), and is involved in a negative feedback loop (Momand et al. 2000). Recently, several reports have revealed that p53 protein is activated through extensive post-translational modifications, including phosphorylation and acetylation, in response to stress signals (Lakin and Jackson 1999). Among several phosphorylation sites, serine residue (Ser) 15 prevents the binding of MDM2 resulting in the alleviation of MDM2-dependent inhibition of p53 activity, whereas Ser 392 stimulates the DNA-binding activity of p53 (Kapoor et al. 2000).

HER2/neu encodes a 185 kD protein which is a member of the membrane-spanning type I receptor tyrosine kinase family (Harari and Yarden 2000). Overexpression of HER2/neu has been found in many types of cancers with high frequency, suggesting its critical role in the development of human tumors (Hung and Lau 1999). Ectopic overexpression of HER2/neu to the high levels observed in some tumors is reported to enhance tumorigenicity in model systems (Di Fiore et al. 1987; Hudziak et al. 1988). HER2/neu is known to activate several signaling pathways, including mitogen-activated protein kinases (MAPK) (Ben Levy et al. 1994) and Phosphatidylinositol-3'-OH kinase (PI3-K) pathways (Peles et al. 1992). Both of these pathways are known to enhance cell proliferation and survival, although MAPK, when activated by some conditions of stress, may mediate apoptosis (Wang et al. 2000).

Therapy of human cancer often includes treatment with cisplatin-based combination regimens (Schiller 2001; du Bois 2001). Although Cisplatin has clinical utility against several tumor types, the presence of primary or the emergence of secondary resistance significantly undermines the curative potential of this drug (Siddik et al. 1999). In view of the central problem of cisplatin resistance, efforts have focused on the development of alternative platinum-based analogues. We have reported the compound 1R,2R-diaminocyclohexane-diacetato-dichloro-platinum (IV) (DACH-acetato-Pt; Figure 1) as a candidate with clinical potential in cisplatin resistance (Kido et al. 1993; Al-Baker et al. 1994) and mechanistic studies have been in progress to rationalize its activity.

Although the role of functional p53 on the sensitivity to cisplatin is still controversial (Fan et al. 1995; Hawkins et al. 1996), it is widely established that cisplatin-induced DNA damage activates signaling pathways culminating in p53 induction (Siddik et al. 1998). Recent reports have shown that the activation of extracellular signal-regulated protein kinase (ERK), which is a member of the MAPK, targets p53 phosphorylation at Ser 15 (Persons et al. 2000) and is required for cisplatin-induced apoptosis (Wang et al. 2000). DACH-acetato-Pt, on the other hand, has shown greater potency against cisplatin-resistant ovarian tumor cells with wild-type p53, but was less cytotoxic against cells having mutant or null p53 (Hagopian et al. 1999). This compound is very efficient in inducing p53 in cisplatin-resistant wild-type p53 ovarian tumor models, and disruption of wild-type p53 function increased resistance to the compound. These facts suggest that the potent antitumor activity of DACH-acetato-Pt is p53-dependent and that different signaling pathways are activated with this platinum agent than with cisplatin.

HER2/neu is also known to induce resistance to some antitumor agents and sensitivity to others (Andersen and Borresen 1995; Pegram et al. 1997). Although the mechanisms of drug resistance induced by HER2/neu are still unclear, there are some reports that suggest an interaction with pathways for p53 and p21 (Bacus et al. 1996; Yu et al. 1998). Recently, Casalini et al. (2001) reported that overexpression of HER2/neu promoted growth inhibition and apoptosis in tumor cells bearing wild-type p53 but was associated with proliferation in cells with mutant p53. It is also reported that ras-mediated signal transduction pathway, which is one of the major down-stream targets of HER2/neu signal, inhibits p53 function (Ries et al. 2000). This pathway is known to play a major role in the expression of resistance to DNA-damaging agents (Dempke et al. 2000). However, the effect of HER2/neu overexpression on p53 induction in response to DNA damage is yet to be clarified.

The purpose of this study is to assess the effects of HER2/neu overexpression on the sensitivity to cisplatin and the novel analog DACH-acetato-Pt in isogenic tumor models with different p53 status. We report herein that increased levels of HER2/neu increased resistance to cisplatin only in cells bearing wild-type p53 but not mutant p53, whereas they increased sensitivity to DACH-acetato-Pt regardless of p53 status. In addition, overexpressed HER2/neu down-regulated p53 by suppressing its phosphorylation at Ser 15 and Ser 392 in cells with wild-type p53 following cisplatin treatment, but did not reduce p53 induction in response to DACH-acetato-Pt.

RESULTS

Status of HER2/neu in MCF-7 and MDA-MB-435 Cells. We have examined the status of HER2/neu in MCF-7 and MDA-MB-435 cell lines. As shown in Figure 2A, the low expression of HER2/neu is apparent in parental and neo cells of both models, whereas MCF-7/HER2-18 and MDA-MB-435/eB1 express substantially high levels of $p185^{HER2/neu}$. The SK-Br3 cell line, with established amplification and overexpression of HER2/neu gene, was used as a positive control. The levels of phophorylated $p185^{HER2/neu}$, p53 and related proteins are shown in Figure 2B. The active, phosphorylated form of $p185^{HER2/neu}$ was substantially higher in HER2/neu transfected cells than in neo-control cells. Interestingly, the basal expression of MDM2 was 1.4-fold higher in MCF-7/HER2-18 cells than that in neo-control cells. On the other hand, basal levels of p53 and p21 were similar in MCF-7/HER2-18 compared to neo, while a modest up-regulation of p21 was observed in MDA-MB-435/eB1 cells, as reported previously (Yu et al. 1998).

Cytotoxicity of Cisplatin and DACH-acetato-Pt. The results of cytotoxic evaluation are shown in Table 1. Increased levels of $p185^{HER2/neu}$ led to an increase in resistance to cisplatin about 2- to 3-fold in wild-type p53 MCF-7 model (IC_{50}'s, 0.34 vs. 0.94 μM for continuous drug exposure and 9.75 vs. 18.2 μM for 2-h exposure, respectively). In contrast, MCF-7/ HER2-18 cells demonstrated significant sensitivity to DACH-acetato-Pt by up to 3-fold compared to neo (IC_{50}'s, 0.055 vs. 0.18 μM for continuous drug exposure and 15.0 vs. 22.1 μM for 2-h exposure, respectively). Although the HER2/neu status did not significantly affect the sensitivity to cisplatin in mutant p53 MDA-MB-435 cells, the cytotoxicity of DACH-acetato-Pt was enhanced in

MDA-MB-435/eB1 cells compared to neo (IC_{50}'s, 1.31 vs. 1.75 μM for continuous drug exposure and 39.9 vs. 68.9 μM for 2-h exposure, respectively). On the other hand, IC_{50} of DACH-acetato-Pt was much higher in mutant p53 MDA-MB-435 cell lines than in wild-type p53 MCF-7 cell lines, suggesting p53-dependent cytotoxicity of this drug, as shown in our previous study (Hagopian et al. 1999).

Biochmical Pharmacology of Cisplatin and DACH-acetato-Pt. The results of biochemical pharmacology are shown in Table 2. Increased levels of $p185^{HER2/neu}$ affected neither cellular platinum uptake nor DNA adduct formation after 2-h exposure to cisplatin or DACH-acetato-Pt in both models. DNA damage tolerance is defined as the level of adducts that are required to kill 50% of the tumor cells. DNA damage tolerance to cisplatin was significantly higher in the MCF-7/HER2-18 than in neo (0.63 vs. 0.20 ng Pt/mg DNA), whereas there was no significant difference in damage tolerance between MDA-MB-435 cells exposed to cisplatin (0.93 vs. 1.1 ng Pt/mg DNA). On the other hand, the increased sensitivity to DACH-acetato-Pt was accompanied by a significant decrease in DNA damage tolerance both in MCF-7 (0.010 vs. 0.027 ng Pt/mg DNA) and in MDA-MB-435 (0.28 vs. 0.38 ng Pt/mg DNA) models. These results suggest that the modulation of cytotoxicity by HER2/neu overexpression on the sensitivity to the platinum agents was due entirely to the change in tolerance to platinum adducts.

Concentration-dependent Induction of Total and Phosphorylated p53 and Transactivation of p21 by Cisplatin and DACH-acetato-Pt. The cytotoxicity and biochemical pharmacology data shown above strongly suggest that the decrease in cisplatin sensitivity by HER2/neu overexpression is p53-dependent, whereas the increased sensitivity to DACH-acetato-Pt by HER2/neu is independent of p53. To examine the role of HER2/neu on the activation of wild-type p53 in response to platinum-induced DNA damage, MCF-7 transfectants were exposed to cisplatin or DACH-acetato-Pt and cellular extracts subjected to Western analysis. Figure 3 shows the dose-dependent induction of p53 and related proteins following exposure to each drug. For both drugs, induction of total p53 is seen to be dependent on concentration both in MCF-7/neo and MCF-7/HER2-18. Although the phosphorylation of p53 at Ser 15 and Ser 392 is apparent with cisplatin treatment, a lower extent of Ser15 and a relatively poor phosphorylation at Ser 392 are observed with DACH-acetato-Pt. Increased levels of $p185^{HER2/neu}$ attenuated phosphorylation of p53 at both Ser 15 and Ser 392 with cisplatin, whereas no difference was apparent between both cell types with DACH-acetato-Pt. The transactivation of p21 is also seen to be dependent on the concentration of cisplatin in both cell types, and the extent is consistent with the levels of phosphorylated p53. In contrast, there is no difference in the transactivation of p21 between cells exposed to DACH-acetato-Pt. The expression of HER2/neu and the active phosphorylated form of $p185^{HER2/neu}$ were not affected by cisplatin or DACH-acetato-Pt at any concentration (data not shown).

Time-dependent Induction of p53 and Associated Proteins. The alterations of protein expression with time following a 2-h exposure to 20 μM drug concentration are shown in Figure 4. Levels of p53 and associated proteins increased with time following drug exposure. The results of densitometric analysis on time-course induction of p53 and p21 are shown in Figure 5. The levels of p53 induced by cisplatin peaked at 36 hr in control MCF-7 cells, and were greatly reduced by HER2/neu overexpression from 24 to 48 hr. The induction kinetics of p53 in response to DACH-acetato-Pt was different than that with cisplatin. By 6 hr, DACH-acetato-Pt had induced p53 in MCF-7/neo cells to levels that approached peak levels observed with cisplatin at 24 hr. However, in the case of DACH-acetato-Pt, a reduction in p53 was not observed by HER2/neu overexpression. Instead, this overexpression appeared to sustain the induction of p53 beyond the 12hr time point. The temporal aspect of transactivation of p21 was consistent with the levels of p53 with both drugs. On the other hand, MDM2 was also transactivated in a p53-dependent manner (Figure 4), but unlike p21, the levels of MDM2 increased more rapidly in MCF-7/HER2-18 cells than in neo cells in response to both drugs. These results suggest that the up-regulation of MDM2 in HER2/neu–transfected cells is promoted by the overexpression of HER2/neu. The extent of phosphorylated p53 at Ser 15 and Ser 392 is shown in Figure 6. Increased levels of HER2/neu significantly suppressed the phosphorylation of p53 at both sites in response to cisplatin-induced DNA-damage, whereas reduced or poor phosphorylations at these p53 sites were seen in both cell types with DACH-acetato-Pt. It is likely that the suppression of p53 induction in MCF-7/ HER2-18 exposed to cisplatin was due to the decreased phosphorylations at the sites.

DISCUSSION

Human tumors rarely possess a single genetic defect, and it is likely that therapeutic outcome following chemotherapy will depend on the relative modulatory effect on each of the molecular targets. In our previous study, we have demonstrated that DACH-acetato-Pt is effective against refractory cancers possessing wild-type p53 (Hagopian et al. 1999). Since such tumors can also demonstrate amplification/overexpression of HER2/neu (Thor et al. 1998), it was important to examine if this molecular defect could influence the potential therapeutic benefit of the platinum analog. On the other hand, several groups have reported that combination of gene therapy using functional p53 with DNA damaging agents may have a synergistic effect without additional toxicity (Fujiwara et al. 1994; Osaki et al. 2000). Consequently, several clinical studies are in progress and platinum drugs have been used as candidates for the DNA damaging agents (Roth et al. 2002). In order to evaluate the possibility of future gene-based therapy against HER2/neu-overexpressing tumors, it is important to also clarify the interaction of p53 and HER2/neu following platinum-induced DNA damage. In this study, we have demonstrated that, unlike cisplatin, the cytotoxicity of DACH-acetato-Pt is increased by HER2/neu overexpression.

The absence of functional p53 in tumor cells is known to be associated with resistance to chemotherapeutic agents (Lowe et al. 1993; 1994). As for cisplatin, however, the implication of functional p53 is unclear. Several studies have shown evidence for positive effects of functional p53 on cisplatin sensitivity. Gallagher et al. (1997) reported that expression of p53 genetic suppressor element decreased p53 protein levels resulting in an 8-fold increase in resistance to cisplatin in A2780 ovarian carcinoma cells. Similarly, Gurnani et al. (1999) introduced adenovirus-mediated wild-type p53 into human cell lines and demonstrated significant increase in cisplatin sensitivity. On the contrary, several groups have reported that inactivation of p53 function with introduction of papilloma virus E6 gene enhanced sensitivity to cisplatin (Fan et al. 1995; Hawkins et al. 1996). Although the role of functional p53 on the sensitivity to cisplatin may depend on cell types, it is well established that cisplatin activates pathways for p53 induction (Siddik et al. 1998; Lakin and Jackson 1999).

One of the recent topics of interest on the regulation of p53 is the post-translational activation of this molecule in response to DNA damage (Albrechtsen et al. 1999; Lakin and Jackson 1999). Phosphorylation and acetylation at several sites have been reported to activate p53 protein. Among these modification sites, Persons et al. (2000) demonstrated that cisplatin-induced DNA damage resulted in phosphorylattion of p53 at Ser15 through activation of ERK-1, 2/MAPK pathway, which appeared to be required in cisplatin-induced apoptosis (Wang et al. 2000). In the present study, we have shown that cisplatin induced phosphorylation of p53 not only at Ser 15 but also at Ser 392. Kapoor et al. (2000) have also reported phosphorylation at both sites in response to UV radiation. They demonstrated that phosphorylation at Ser 15 was implicated in stabilization of p53, while phosphorylation at Ser392 (Ser 389 in mouse) increased its DNA-binding activity. Thus, cooperative phosphorylation at these sites was reported to activate p53 effectively (Kapoor et al. 2000). Consistent with the results from UV radiation, our results revealed that phosphorylation of these two sites contributed to both the stabilization and increased transactivational activity of p53 in response to cisplatin DNA damage. On the other hand, a lesser extent of Ser15 phosphorylation and a very poor phosphorylation at Ser 392 were observed with DACH-acetato-Pt, suggesting that different mechanism of post-translational modifications is involved in the activation of p53 in response to DACH-acetato-Pt. This pattern of p53 modification is analogous to that reported by ionizing radiation (Kapoor and Lozano 1998), and may be critical in the effectiveness of the analog against cisplatin resistant tumor cells.

A significant body of evidence on chemoresistance induced by HER2/neu has been reported in experimental studies as well as in clinical studies (Harari and Yarden 2000; Pegram et al. 1997). In this study, we have shown that increased levels of HER2/neu led to a 2- to 3-fold increase in cisplatin resistance in wild-type p53 MCF-7 model, whereas it did not affect the sensitivity to cisplatin in mutant p53 MDA-MB-435 model. Pegram et al. (1997) have reported the effect of HER2/neu overexpression on chemotherapeutic drug sensitivity in human breast and ovarian cancer cells. Although the authors did not focus on p53 status, overexpression of HER2/neu significantly decreased the sensitivity to cisplatin of their two tumor cell lines with wild-type p53, including MCF-7. In contrast, there was no significant difference in sensitivity to cisplatin in 3 out of the 4 cell lines with mutant p53, including MDA-MB-435. These findings are consistent with our results, suggesting that HER2/neu-induced resistance to cisplatin is p53-dependent. On the other hand, HER2/neu-transfected cells showed significant sensitivity to the novel platinum analog DACH-acetato-Pt regardless of p53 status. In addition, we have also shown that the modulation of cytotoxicity by HER2/neu was due entirely to the change in tolerance to platinum-induced DNA damage. These data suggest that overexpression of HER2/neu results in greater tolerance to DNA-platinum adducts through down-regulation of signaling pathways to p53 when cisplatin is the DNA damaging agent, whereas it increased the sensitivity to DACH-acetato-Pt through a reduction in adduct tolerance, which was independent of the p53 status.

An important fact we have uncovered in the present study is that increased levels of HER2/neu attenuate the phosphorylation of p53 both at Ser15 and Ser392 following cisplatin-induced DNA damage in wild-type p53 cell lines. As a result, both p53 induction and the p21 product from its transcriptional activity were significantly suppressed in MCF7/HER2-18 cells compared to control cells. Although the precise mechanism of how HER2/neu down-regulates p53 remains unclear, an explanation to account for this may be realized by considering the upregulation of MDM2 levels observed in MCF-7/HER2-18 cells. MDM2 binds p53 close to its N-terminus and acts as an ubiquitin ligase (Honda et al. 1997; Lakin and Jackson 1999), and phosphorylation of p53 at Ser 15 is known to inhibit binding of MDM2 to p53 and blocks MDM2-mediated degradation of p53 (Shieh et al. 1997). Recently, Zhou et al. (2001) reported that HER2/neu induced MDM2 phosphorylation at Ser 166 and Ser 186 through PI3-K pathways. According to their model, these phosphorylations of MDM2 enhance its nuclear localization, which increases p53 ubiquitination via its interaction with the transcriptional co-activator CPB/p300. This model strongly supports our findings with cisplatin.

In contrast, increased levels of HER2/neu did not reduce the induction of p53 in response to DACH-acetato-Pt. Instead, overexpression of HER2/neu sustained the induction of p53 after the 12hr time point, suggesting a positive effect of $p185^{HER2/neu}$ signaling on the sensitivity to DACH-aceato-Pt. Moreover, HER2/neu enhanced the cytotoxicity of DACH-acetato-Pt regardless of p53 status, although DACH-acetato-Pt was much more potent against wild-type p53 MCF-7 cells than against mutant p53 MDA-MB-435 cells. These results, demonstrating a dependency on p53 status for potency, are consistent with our previous report using ovarian tumor models (Hagopian et al. 1999). These findings indicate that introduction of functional p53 in combination with DACH-acetato-Pt may be an effective treatment against cells lacking functional p53 and overexpressing HER2/neu.

In this study, we have demonstrated that overexpression of HER2/neu leads to cisplatin resistance by down-regulating p53 in wild-type p53 cell lines, but it dose not affect the sensitivity in mutant p53 cell lines. HER2/neu overexpression, on the other hand, increases the sensitivity to DACH-acetato-Pt independent of p53. These results suggest that cisplatin and DACH-acetato-Pt activate independent signal transduction pathways that regulate p53, and that HER2/neu impinges only on the pathway activated by cisplatin. Therefore, we postulate that DACH-acetato-Pt may have clinical utility in the management of tumors overexpressing HER2/neu, particularly against a wild-type p53 background from potency consideration.

MATERIALS AND METHODS

Chemicals. Cisplatin was obtained from Sigma Chemical Co. (St. Louis, MO). We have previously reported the synthesis and chemical characterization of DACH-acetato-Pt (Al-Baker et al. 1994). Cisplatin and DACH-acetato-Pt were dissolved in normal saline and water, respectively, then sterilized through 0.22-µm disc filters. The concentration of each drug was confirmed by flameless atomic absorption spectroscopy (FAAS) (Siddik et al. 1987). MTT was purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Lines. MCF-7/HER2-18 having wild-type p53 and MDA-MB-435/eB1 with mutant p53, both of which are stably transfected with full-length HER2/neu cDNA, and control isogenic neo cell lines have been described previously (Yu et al. 1998; Benz et al. 1993). The cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine and antibiotics under a humidified atmosphere of 5 % CO_2.

Cytotoxicity and Biochemical Pharmacology Studies. For cytotoxic determinations, 500 to 1000 cells were plated in 100 µl of medium in 96-well plates. Following 2 days of incubation, cells were exposed to various concentrations of cisplatin or DACH-acetato-Pt. After another 4 or 5 days, the relative sensitivities of the cells to the platinum complexes were evaluated using a modified MTT assay (Carmichael et al. 1987). Evaluations in attached cells of cellular platinum uptake and DNA adduct formation were conducted as described previously (Kido et al. 1993; Yoshida et al. 1994). Briefly, cells treated with cisplatin or DACH-acetato-Pt (100 or 200 µM) for 2 h at 37 °C were microfuged and washed. For determination of cellular uptake, aliquots of cell pellets were digested overnight at 55 °C in 50 μl of 1 M hyamine hydroxide (ICN, Irvine, CA). To measure platinum-DNA adduct formation, high molecular weight DNA was isolated from cell pellets according to standard procedures (Maniatis et al. 1982). The platinum content of samples was determined by FAAS. Platinum-DNA damage tolerance was defined as the value of DNA adducts at an IC_{50} concentration (Johnson et al. 1997).

Western Analysis Cells were exposed for 2 h to various concentration of cisplatin or DACH-acetato-Pt, washed, and incubated at 37 °C in drug-free medium for indicated time. For dose-dependent analysis, the cells were harvested after 24 h incubation. For time-course study, cells were exposed to 20 μM of each drug. The cells were then washed twice with ice-cold phosphate-buffered saline and lysed for 20 min on ice with 100 μl of lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 100 μg/ml phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin). The lysates were collected by microcentrifugation at 4 °C, and then the protein was determined by the standard Lowry procedure. Forty μg of total cell protein was electrophoresed on a 7.5% (for HER2/neu) or 10% (for p53, p21, MDM2) SDS-polyacrylamide gel, transferred onto nitrocellulose membranes, and incubated with various antibodies. Mouse monoclonal anti-p53 (DO-1), anti- MDM2 (Ab-1) and anti-HER2/neu (c-neu, Ab-3) antibodies were obtained from Oncogene Research Products (Cambridge, MA), and anti-p21 (Cip1/Waf1) antibody was purchased from Transduction Laboratories (Lexington, KY). Rabbit polyclonal anti-phospho-HER2/neu (Y1248) antibody was obtained from Upstate Biotechnology (Lake Placid, NY) and Rabbit polyclonal anti-phospho-p53 antibodies (Ser 15 and Ser 392) were purchased from Cell Signaling Technology (Beverly, MA). Mouse monoclonal anti-β-actin antibody was purchased from Sigma (St. Louis, MO). All immunoblots were visualized by enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL), and quantified by laser densitometry.

Statistical Analysis. Differences between groups were evaluated by paired Student's *t* test in the cytotoxicity assays and the biochemical pharmacology and by unpaired *t* test in the DNA-damage tolerance, respectively.

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FIGURE LEGENDS

Figure 1. Structures of cisplatin and DACH-acetato-Pt (IV).

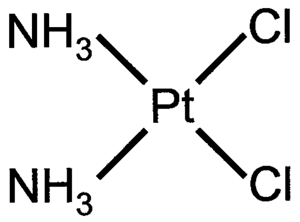
Figure 2. A. Western immunoblot of basal levels of $p185^{HER2/neu}$ in MCF-7 and MDA-MB-435 transfection models. SK-Br3 cells were used as a positive control. B. Basal evpression levels of phosphorylated $p185^{HER2/neu}$, p53 and related proteins.

Figure 3. Concentration-dependent induction of total and phosphorylated p53 and transactivation of p21 in MCF-7 cell lines exposed to cisplatin or DACH-acetato-Pt. Cells were exposed to various concentrations of drugs for 2h and then incubated in drug-free medium. Cells were harvested 24 h later, and protein was extracted and sujected to Western analysis.

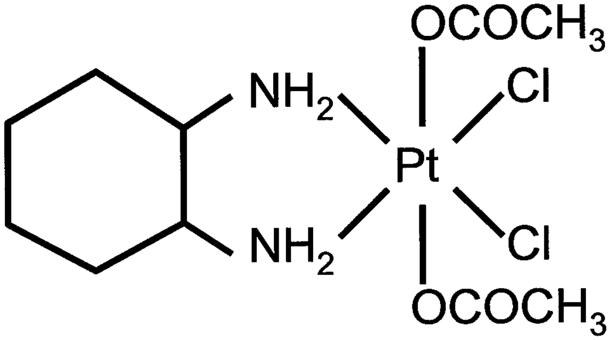
Figure 4. Time-dependent induction of p53 and associated proteins in MCF-7 models after treatment with cisplatin or DACH-acetato-Pt. Cells were treated with 20 μM of drugs for 2-h and subsequently incubated without drug for indicated time. Cells were harvested, and protein was extracted and examined for p53 and associated proteins by Western analysis.

Figure 5. Levels of p53 and p21 during the time-course estimated from immunoblots by laser densitometry.

Figure 6. Levels of phosphorylated p53 at Ser 15 and Ser 392 during the time course estimated from immunoblots by laser densitometry.


NH3
Cl
Pt
NH3
Cl

Cisplatin


OCOCH3
NH2
Cl
Pt
NH2
Cl
OCOCH3

1R,2R-DACH-$(Ac)_2Cl_2$-Pt(IV)

Figure 1

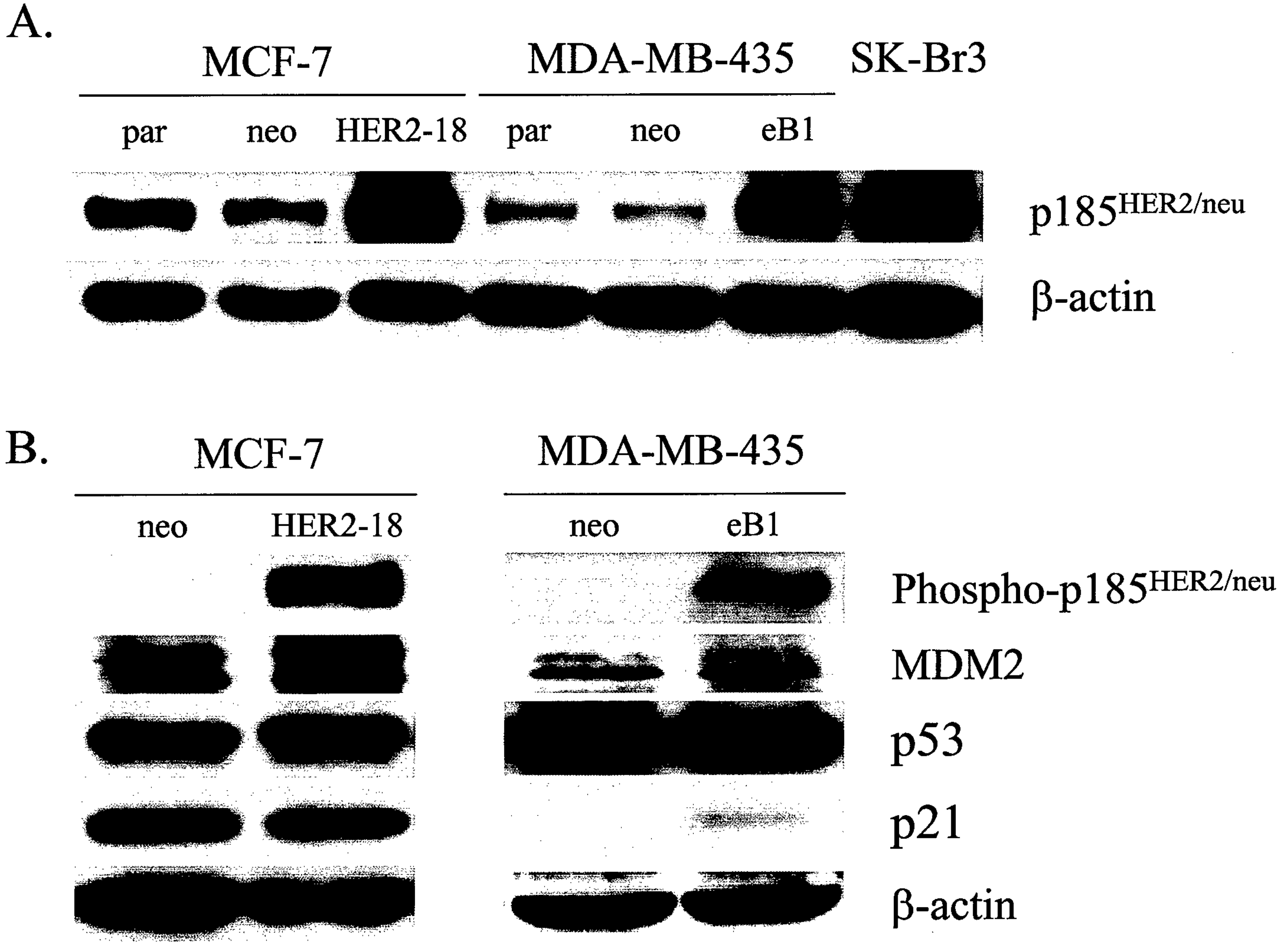
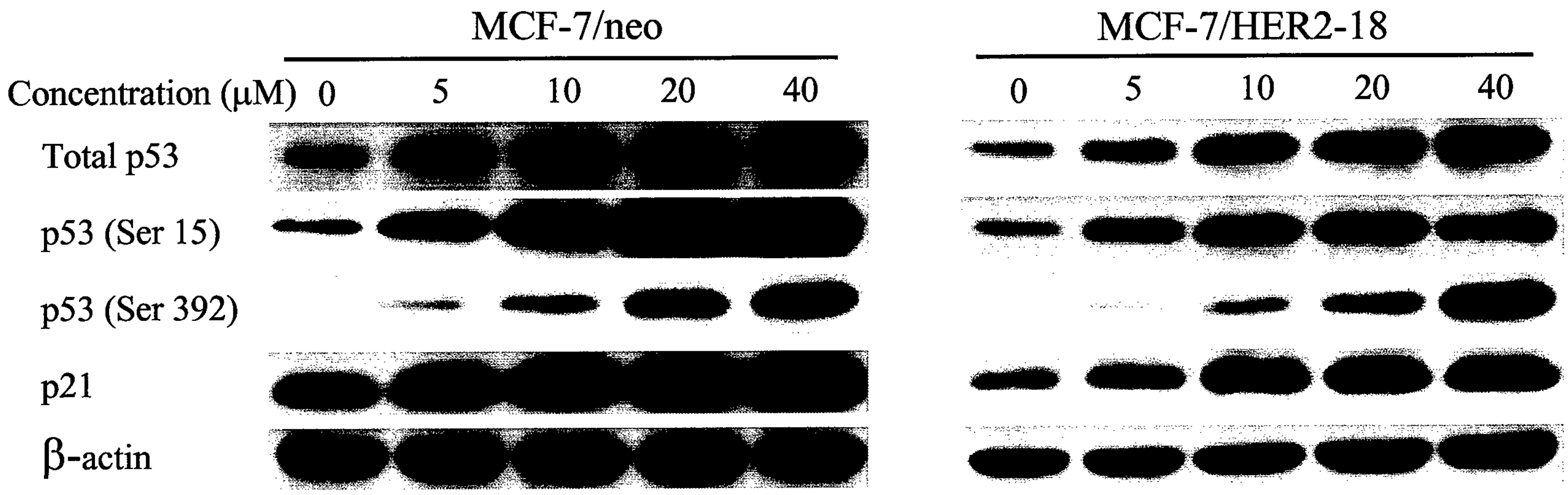

A.
MCF-7
MDA-MB-435
SK-Br3
par
neo
HER2-18
par
neo
eB1
p185^HER2/neu
β-actin
B.
MCF-7
MDA-MB-435
neo
HER2-18
neo
eB1
Phospho-p185^HER2/neu
MDM2
p53
p21
β-actin

Figure 2

A. Cisplatin


MCF-7/neo
MCF-7/HER2-18
Concentration (μM)
0
5
10
20
40
0
5
10
20
40
Total p53
p53 (Ser 15)
p53 (Ser 392)
p21
β-actin

B. DACH-acetato-Pt

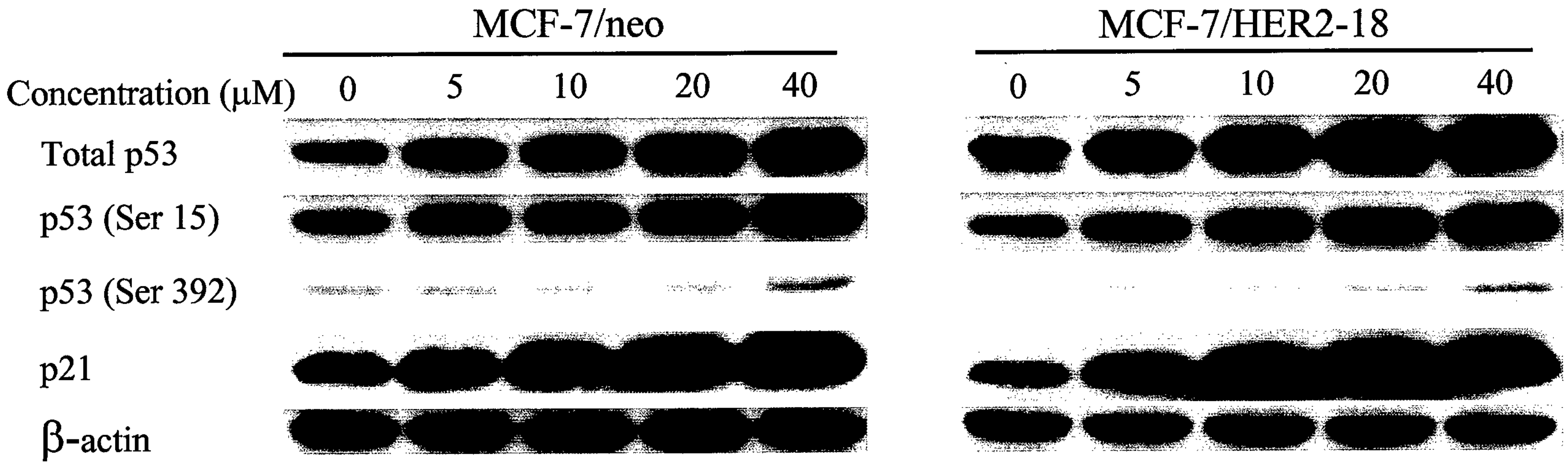
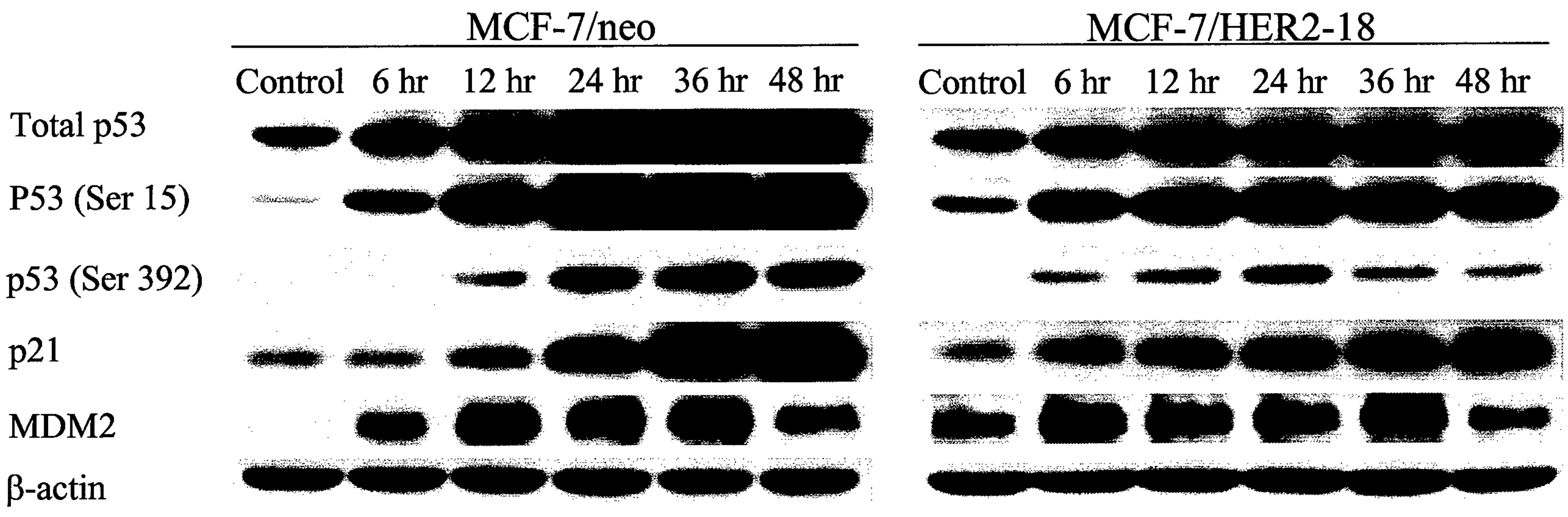

MCF-7/neo
MCF-7/HER2-18
Concentration (μM)
0
5
10
20
40
0
5
10
20
40
Total p53
p53 (Ser 15)
p53 (Ser 392)
p21
β-actin

Figure 3

A. Cisplatin


MCF-7/neo
MCF-7/HER2-18
Control
6 hr
12 hr
24 hr
36 hr
48 hr
Total p53
P53 (Ser 15)
p53 (Ser 392)
p21
MDM2
β-actin

B. DACH-acetato-Pt

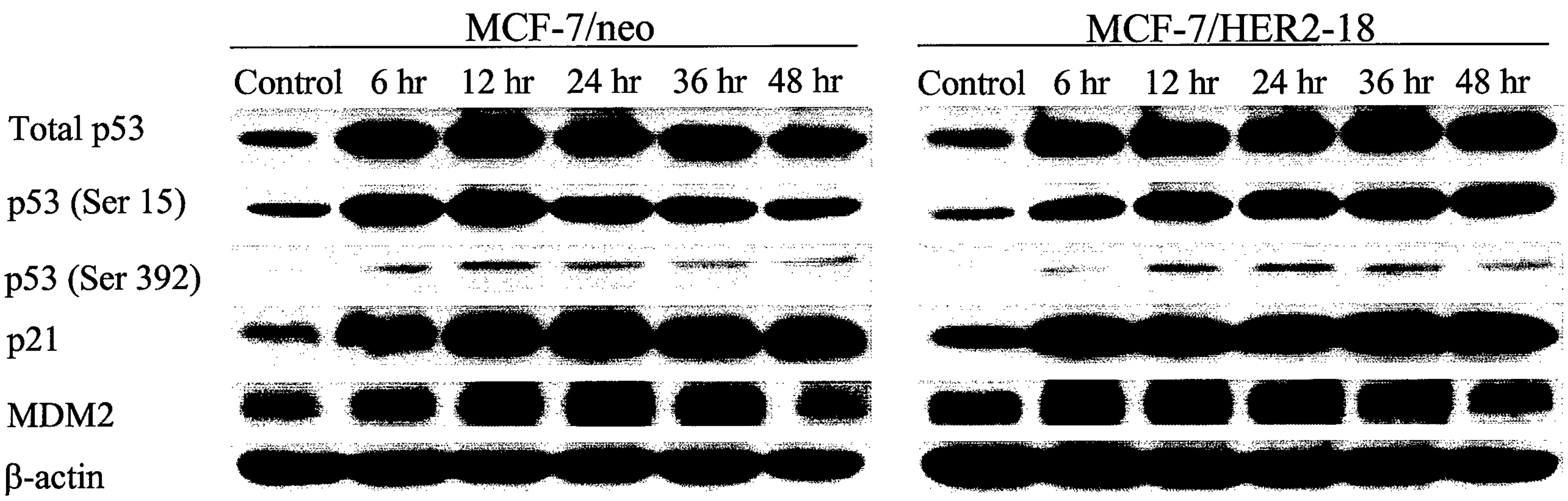
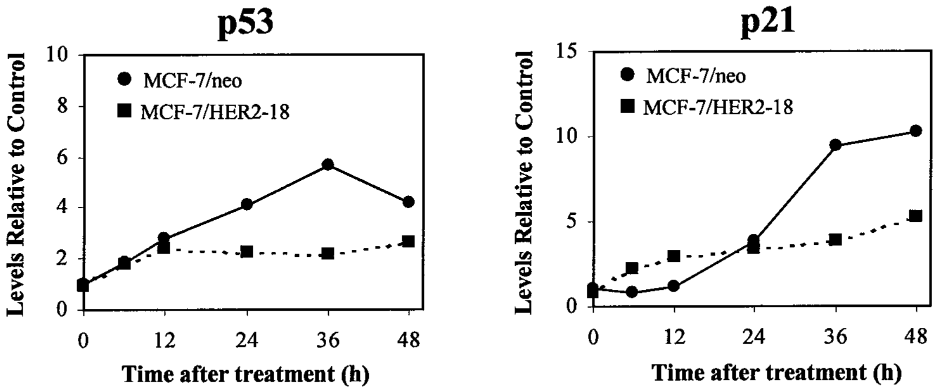

MCF-7/neo
MCF-7/HER2-18
Control
6 hr
12 hr
24 hr
36 hr
48 hr
Total p53
p53 (Ser 15)
p53 (Ser 392)
p21
MDM2
β-actin

Figure 4

A. Cisplatin



B. DACH-acetato-Pt

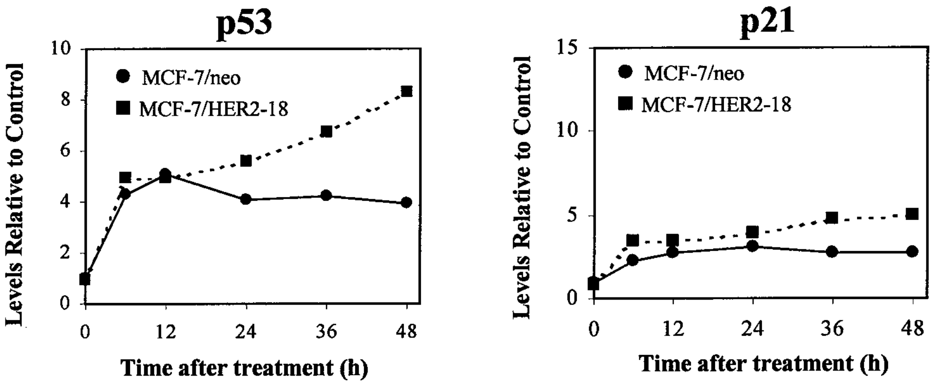


Figure 5

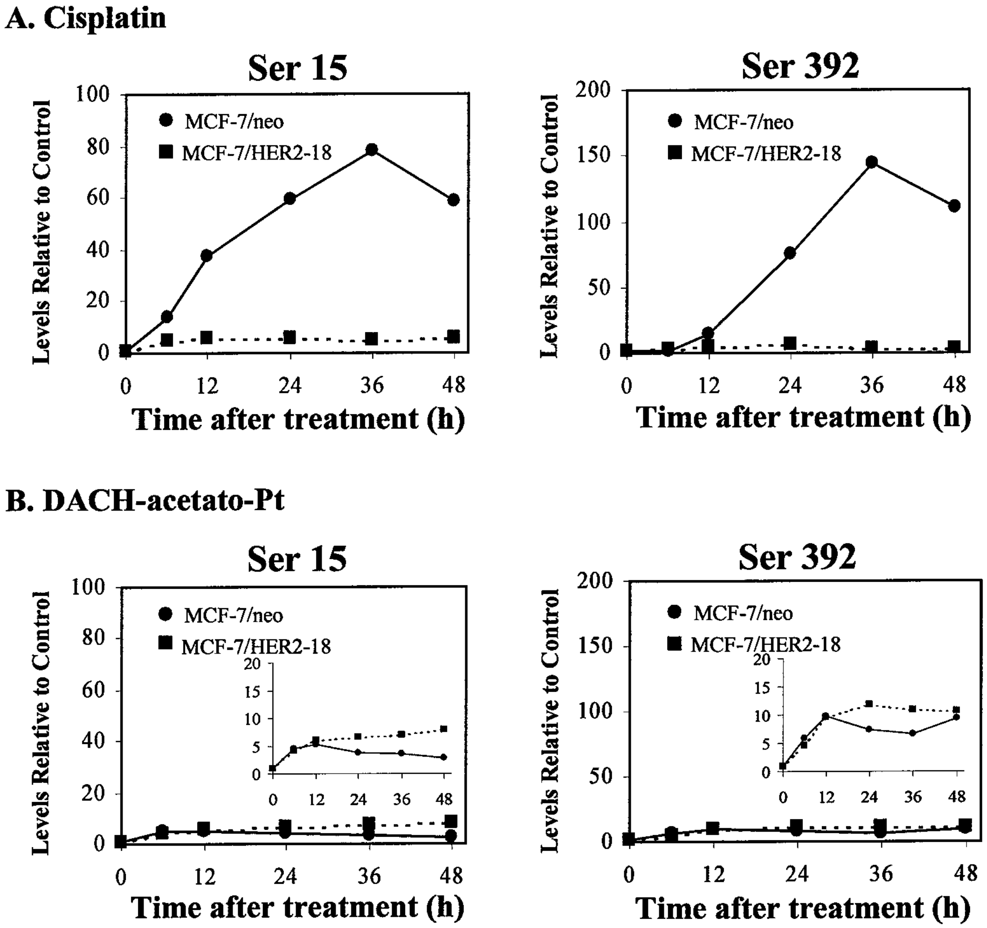

A. Cisplatin
Ser 15
Ser 392
MCF-7/neo
MCF-7/HER2-18
Levels Relative to Control
100
80
60
40
20
0
200
150
100
50
0
0
12
24
36
48
Time after treatment (h)
B. DACH-acetato-Pt
Ser 15
Ser 392
20
15
10
5

Figure 6

Table 1. Effect of HER2/neu overexpression on the cytotoxicity of cisplatin and DACH-acetato-Pt following continuous or 2-h drug exposure

	Continuous drug exposure		2-h drug exposure	
Cell line	Cisplatin	DACH-acetato-Pt	Cisplatin	DACH-acetato-Pt
MCF-7/neo	0.34 ± 0.04*	0.18 ± 0.08	9.75 ± 1.73	22.1 ± 4.0
MCF-7/HER2-18	0.94 ± 0.31**	0.055 ± 0.016**	18.2 ± 4.9**	15.0 ± 3.3**
MDA-MB-435/neo	1.41 ± 0.33	1.75 ± 0.41	5.63 ± 0.52	68.9 ± 8.2
MDA-MB-435/eB1	1.15 ± 0.12	1.31 ± 0.34**	4.99 ± 0.30	39.9 ± 1.0**

*Mean ± SD; **$P<0.05$, vs. neo with paired t-test; n=3-5

Table 2. Biochemical pharmacology of cisplatin and DACH-acetato-Pt against MCF-7 and MDA-MB-435 transfectants

	Cellular uptake (ng platinum/mg protein)		DNA adduct formation (ng platinum/mg DNA)		DNA-damage tolerance (ng platinum/mg DNA)	
Cell line	Cisplatin	DACH-acetato-Pt	Cisplatin	DACH-acetato-Pt	Cisplatin	DACH-acetato-Pt
MCF-7/neo	145.4 ± 21.4*	55.6 ± 9.1	59.8 ± 17.7	15.1 ± 4.5	0.20 ± 0.03	0.027 ± 0.012
MCF-7/HER2-18	149.9 ± 42.5	71.7 ± 17.6	66.5 ± 13.8	17.9 ± 3.9	0.63 ± 0.21**	0.010 ± 0.003**
MDA-MB-435/neo	217.4 ± 28.0	142.1 ± 38.4	75.2 ± 13.5	21.5 ± 5.0	1.1 ± 0.3	0.38 ± 0.09
MDA-MB-435/eB1	266.7 ± 87.2	120.1 ± 37.9	81.2 ± 7.7	21.1 ± 5.4	0.93 ± 0.10	0.28 ± 0.07**

*Mean ± SD; **$P<0.05$, vs. neo with unpaired *t*-test; n=3-6

A novel analog 1R,2R-Diaminocyclohexane-diacetato-dichloro-Pt (IV) circumvents cisplatin resistance induced by upregulation of p21$^{waf1/cip1}$ in breast cancer cell lines[1]

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Running Title: p21 and platinum resistance

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INTRODUCTION

Breast cancer is the most frequently diagnosed malignancy in American women, and the second most common cause of cancer death. In 2002, it was predicted that an estimated 203,500 new cases would be diagnosed of this disease and approximately 39,600 would die in the United States {Jemal, Thomas, et al. 2002 34 /id}. Although approximately 80% of breast cancer patients present with disease limited to the breast and/or axillary lymph nodes, almost half of these patients later develop metastatic disease and eventually succumb to it{Fornier, Munster, et al. 1999 35 /id}. Therefore, systemic therapy, including chemotherapy and hormonal manipulation, is in a great position for the management of breast cancer patients, especially with advanced or metastatic diseases, while surgical and radiotherapeutic interventions contribute to local control.

Cisplatin is among the most widely used and broadly active antitumor drugs {du 2001 54 /id}{Schiller 2001 53 /id}. Despite its wide spectrum of clinical activity, however, cisplatin has not been used for the treatment of breast cancer patients. One of the major reasons is that early clinical studies with heavily pretreated patients demonstrated little or no activity {Yap, Salem, et al. 1978 38 /id}. On the contrary, several groups have later revealed that the overall response rate among patients with advanced breast cancer given high-dose cisplatin without prior chemotherapy was 42 –54 % {Sledge, Loehrer, et al. 1988 40 /id}{Kolaric & Roth 1983 39 /id}, suggesting that cisplatin may be an effective agent against this disease. More recently, several phase studies using cisplatin combined with anthracyclines {Nielsen, Dombernowsky, et al. 2000 41 /id}or taxanes {Rosati, Riccardi, et al. 2000 42 /id} have indicated a very high potential of cisplatin in the combination setting for the treatment of patients with advanced or metastatic breast cancer. In spite of the increased evidence for the clinical utility of cisplatin for breast cancer patients, the response rates of the combination are not durable and do not translate into an improved 5-year survival rate. This is likely attributable to the development of drug resistance, which is a persuasive factor in the quest of novel agents.

The presence of primary or the emergence of acquired resistance to cisplatin is indeed a major limitation in medical oncology {Siddik, Hagopian, et al. 1999 18 /id}. Several mechanisms have been implicated for this, including decreased drug accumulation, increase in thiol levels and increased DNA repair {Timmer-Bosscha, Mulder, et al. 1992 36 /id}. In addition to these classical resistance mechanisms, there is considerable evidence to show that genetic alterations in tumors, including activation of oncogenes and inactivation of tumor suppressor genes, could modify the cisplatin-induced signaling pathways, and thereby increase the tolerance to DNA damage {Dempke, Voigt, et al. 2000 30 /id}. Since Burchenal et al. demonstrated that specific platinum analogs could circumvent cisplatin resistance, efforts have been made to identify alternative platinum analogs that are effective against resistant tumors {Burchenal, Kalaher, et al. 1979 38 /id}. In this regard, we have reported that the analog 1R,2R-diaminocyclohexane-diacetato-dichloro-platinum (IV) (DACH-acetato-Pt, Figure1) is a candidate with clinical potential in cisplatin resistance {Al-Baker, Siddik, et al. 1994 31 /id}.

DACH-acetato-Pt appears to have greater potency against cisplatin-resistant ovarian tumor cells with wild-type p53, but was less toxic against cells having mutant or null p53 {Hagopian, Mills, et al. 1999 45 /id}. Moreover, this compound is very efficient in inducing p53 in cisplatin-resistant wild-type p53 tumor models, and disruption of p53 function increased resistance to the compound. We have proposed that DACH-acetato-Pt and cisplatin activate independent signaling pathways for p53 induction, and this characteristic may be significant in the potential management of cisplatin-resistant ovarian tumors, particularly against a wild-type p53 background {Hagopian, Mills, et al. 1999 45 /id}. Breast and ovarian cancers are known to develop similar genetic impediments, such as mutation/deletion of p53, amplification/overexpression of HER2/neu, loss of BRCA function {Welcsh & King 2001 37 /id}, and estrogen-dependency. As it is feasible that DACH-acetato-Pt may have similar modulatory effect on each molecular targets in both disease types, the novel platinum analog may have a potential against breast tumors in addition to that proposed for ovarian cancer.

In this study, we have compared the cytotoxicity of cisplatin and DACH-acetato-Pt in a panel of breast cancer cell lines and have examined the basis for the relative sensitivity of the tumor panel toward each agents. We report here that the basal levels of universal cyclin-dependent kinase (cdk) inhibitor $p21^{Waf1/Cip1}$ correlated with the resistance to cisplatin, while the sensitivity to DACH-acetato-Pt depended on the presence of functional p53 but was not affected by the upregulation of p21.

MATERIALS AND METHODS

Chemicals. Cisplatin was obtained from Sigma Chemical Co. (St. Louis, MO). We have previously reported the synthesis and chemical characterization of DACH-acetato-Pt {Al-Baker, Siddik, et al. 1994 31 /id}. Cisplatin and DACH-acetato-Pt were dissolved in normal saline and water, respectively, then sterilized through 0.22-µm disc filters. The concentration of each drug was confirmed by flameless atomic absorption spectroscopy (FAAS) {Siddik, Boxall, et al. 1987 32 /id}. MTT was purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Lines. The nine human breast cancer cell lines used in this study were obtained from American Type Culture Collection (ATCC, Rockville, MD). Cells were grown as monolayers in 5% CO_2 and 95% humidified air at 37 °C. The MCF-7, HCC1937, MDA-MB-435, MDA-MB-436 and T47D cell lines were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine and antibiotics. The ZR75-1 and MDA-MB-157 cell lines were grown in Dulbecco's modified Eagle's medium, 10% heat-inactivated FBS, 2 mM L-glutamine, 10 µg/ml Insulin and antibiotics. An almost identical medium, containing 20% heat-inactivating FBS, was used to grow the MDA-MB-330 cell line. The SK-Br3 was maintained in McCoy's 5a medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine and antibiotics.

Analysis of p53 status by Wave analysis and DNA sequencing. Mutation of p53 in ZR75-1 and MDA-MB-330 cell lines was screened by the denaturing high-performance liquid chromatography (DHPLC) technique {Gross, Kiechle, et al. 2001 39 /id}, and suspected exons were subjected to DNA sequencing for confirmation of mutation. Briefly, primer sets spanning exon 5 to exon 10 of p53 were designed and appropriate heteroduplex detection in DHPLC analysis was designed. DHPLC analysis was performed on a Wave DNA Fragment Analysis System (Transgenomic, San Jose, CA) as previously described {Gross, Arnold, et al. 1999 40 /id}. PCR products demonstrating heteroduplex formation were subjected to sequence analysis.

Cytotoxicity and Biochemical Pharmacology Studies. For cytotoxic determinations, 500 to 1000 cells were plated in 100 μl of medium in 96-well plates. Following a 24-hour incubation, cells were exposed continuously to various concentrations of cisplatin or DACH-acetato-Pt. After another 7 days for the MDA-MB-330 cell line and 5 days for the other cell lines, the relative sensitivities of the cells to the platinum complexes were evaluated using a modified MTT assay {Carmichael, DeGraff, et al. 1987 34 /id}. Evaluations in attached cells of cellular platinum uptake and DNA adduct formation were conducted as described previously {Yoshida, Khokhar, et al. 1994 35 /id} {Kido, Khokhar, et al. 1993 19 /id}. Briefly, cells treated with 100 μM cisplatin or DACH-acetato-Pt for 2 h at 37 °C were microfuged and washed. For determination of cellular uptake, aliquots of cell pellets were digested overnight at 55 °C in 50 μl of 1 M hyamine hydroxide (ICN, Irvine, CA). To measure platinum-DNA adduct formation, high molecular weight DNA was isolated from cell pellets according to standard procedures {Maniatis, Frisch, et al. 1982 36 /id}. The platinum content of samples was determined by FAAS. Platinum-DNA damage tolerance was defined as the level of DNA adducts formed at an IC_{50} concentration {Johnson, Laub, et al. 1997 37 /id}

Western Analysis Cells were exposed to various concentration of cisplatin or DACH-acetato-Pt for the indicated length of time. For dose-dependent analysis, the cells were harvested after 24 h incubation. For time-course study, cells were exposed to 10 μM of each drug, washed twice with ice-cold phosphate-buffered saline (PBS), and lysed for 20 min on ice with 100 μl of lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 100 μg/ml phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin). The lysates were collected by microcentrifugation at 4 °C, and the protein determined by the standard Lowry procedure. Forty μg of total cell protein was electrophoresed on a 10% SDS-polyacrylamide gel, transferred onto nitrocellulose membranes, and incubated with various antibodies. Mouse monoclonal anti-p53 (Ab-6, OP43) and anti-p185$^{HER2/neu}$ (Ab-3, OP15) antibodies, and Rabbit polyclonal anti-cyclin E (PC438) were obtained from Oncogene Research Products (Cambridge, MA), and anti-p21 (Cip1/Waf1, C24420) antibody was purchased from Transduction Laboratories (Lexington, KY). Rabbit polyclonal anti-cyclin D1 (sc-718) and anti-p27 (kip1, #06-455) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and Upstate Biotechnology (Lake Placid, NY), respectively. Mouse monoclonal anti-β-actin antibody was purchased from Sigma (St. Louis, MO). All immunoblots were visualized by enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL), and quantified by laser densitometry.

Cell cycle analysis. Attached cells in an exponential growth phase in 100-mm tissue culture dishes were exposed for upto 48 h to various concentrations of cisplatin or DACH-acetato-Pt. The cells were collected, washed twice with ice-cold PBS, and fixed with a 1% final concentration of paraformaldehyde in PBS for 15 minutes on ice. The cells were then washed with ice-cold PBS, resuspended in 70% ethanol, and stored at -

20°C. Before analysis, the cell suspension was thawed, washed once with ice-cold PBS, resuspended in a solution of propidium iodide (10 μg/ml) in PBS containing 0.5% Tween 20 and 500 units/ml of RNase A (Sigma) and incubated at room temperature for 30 minutes and then at 4°C overnight. Cell cycle kinetics were determined on a Coulter Epics XL-MCL flow cytometer (Beckman Coulter Inc, Fullerton, CA) and analyzed by Multicycle Software (Phoenix Flow Systems, San Diego, CA).

RESUTLS

p53 status of breast cancer cell lines. p53 status of the nine breast cancer cell lines is shown in Table 1. There were two cell lines with wild-type p53, five with mutant p53 and two demonstrating no expression of p53. Among the mutant p53 cell lines, HCC1937 is unique in that it has mutation leading to termination at codon 306 in exon 8 {Tomlinson, Chen, et al. 1998 41 /id}, which resulted in a truncated p53 protein with an intact DNA binding domain and without any change in the amino acid sequence (Figure 2A). Although this type of mutant p53 is known to have 10 to 100 times lower affinity for DNA, such p53 variants are still able to bind DNA and stimulate transcription {Balagurumoorthy, Sakamoto, et al. 1995 42 /id}. In fact, the basal level of p21 in HCC1937 was similar to that seen in wild-type p53 MCF-7 cell line, and p21 was transactivated in response to both platinum agents in the HCC1937 cell line (data not shown). Based on the above evidence, the HCC1937 cells were grouped with cell lines having functional p53 in the following studies.

Cytotoxic evaluation of cisplatin and DACH-acetato-Pt in breast cancer cell lines. The results from the cytotoxic evaluation of platinum analogs in the panel of breast cancer cell lines are shown in Table 2. In order to evaluate the effect of wild-type p53 on the sensitivity to the platinum agents, the panel of cell lines was divided into two groups according to the status of functional p53. There was no difference in the median IC_{50} of cisplatin between the groups (~0.8 μM in bothe groups). On the other hand, the group with functional p53 demonstrated a lower median IC_{50} value for DACH-acetato-Pt than that without functional p53 (0.23 vs. 1.1-1.3 μM). The cisplatin/DACH-acetato-Pt potency ratio indicates a higher median value for the group possessing wild-type p53 function compared to that without this function (4.9 vs. 0.6-0.7). These results suggest that the potent cytotoxicity of DACH-acetato-Pt against breast cancer cell lines is also wild-type p53-dependent, as was shown in our previous study with the ovarian cancer panel {Hagopian, Mills, et al. 1999 45 /id}.

Biochemical pharmacology of cisplatin and DACH-acetato-Pt against ZR75-1 and T47D breast cancer cell lines. In our previous report, we demonstrated that an increase in DNA damage tolerance was a major mechanism of cisplatin resistance {Siddik, Mims, et al. 1998 52 /id}. In order to understand the biochemical pharmacologic basis of cisplatin resistance and sensitivity to DACH-acetato-Pt, particularly that of the mutant p53 T47D model, we have investigated drug uptake, DNA adducts and tolerance to DNA damage in wild-type p53 ZR75-1 and mutant p53 T47D cell lines. The results of the biochemical pharmacology study are shown in Table 3. The extent of platinum uptake was similar in both cell lines for each drug. DNA adducts in cells exposed to cisplatin were 1.5- to 2-fold higher than those in cells exposed to DACH-acetato-Pt. Thus the increased sensitivity of DACH-acetato-Pt relative to cisplatin was not due to either greater intracellular uptake or formation of the cytotoxic DNA adducts. The tolerance to cisplatin-induced DNA damage, on the other hand, was much higher than that induced by DACH-acetato-Pt in both cell lines, suggesting that relative potency of the two agents is linked to relative tolerance of the cell lines to DNA damage.

Basal expression levels of p185$^{HER2/neu}$, p53 and G1 cell cycle regulators. In order to determine the molecular factor which affects the cytotoxicity of cisplatin, basal expression levels of p185$^{HER2/neu}$, p53 and G1 cell cycle protein were investigated in the breast cancer cell lines (Figure 2B). Consistent with the literature, basal expression of mutant p53 was much higher than p53 in the functionally-competent group. p185$^{HER2/neu}$ was highly overexpressed in MDA-MB-330 and SK-Br3 cell lines, moderately overexpressed in ZR75-1, HCC1937 and T47D cell lines and not overexpressed in the other four cell lines. Cells containing functional p53 (MCF-7, ZR75-1 and HCC1937) expressed higher basal levels of p21 when compared to cells without functional p53, as has been reported previously {El Deiry, Tokino, et al. 1995 44 /id} {El Deiry, Harper, et al. 1994 43 /id}. ZR75-1 expressed p21 levels that were about 3.5-fold higher than MCF-7, whereas HCC1937 cells exhibited a level that was 25% lower than in the MCF-7 model. Among the group of cell lines without functional p53, basal levels of p21 were only observed in MDA-MB-330, T47D and MDA-MB-157 cells, and relative expression levels compared to MCF-7 were 0.24-, 0.11-, and 0.05-fold, respectively. The other regulators of G1/S phase of the cell cycle (p27, Cyclin D1 and Cyclin E) were upregulated in unison in some cell lines, independent of p53 function. Note the multiple bands of Cyclin E, which has been reported previously {Gray-Bablin, Zalvide, et al. 1996 53 /id}.

Correlation between the cytotoxicity of cisplatin and relative expression levels of p21. We have examined the correlation between the markers examined in Figure 2B and IC_{50} values of the platinum analogs, and found that the basal expression levels of p21 loosely correlated with the relative sensitivity to in both functional p53-positive and -negative groups (Figure 3). In contrast, the correlation between relative p21 expression and IC_{50} values for DACH-acetato-Pt was not significant.

Induction of p53 and transactivation of p21 in breast cancer cell lines. In order to evaluate the role of p53 in response to the platinum drugs, cells were treated with

5 x IC_{50} concentrations of cisplatin or DACH-acetato-Pt for 24 h, and the cell extracts were subjected to Western immunoblot analysis (figure 4). The levels of p53 were increased in both wild-type p53 cell lines, whereas no significant inducion of p53 was seen in cell lines with either truncated or mutant p53. In the three cell lines with functional p53 (MCF-7, ZR75-1 and HCC1937), p21 was transactivated following exposure to DACH-acetato-Pt, whereas no significant increase in levels of p21 was observed after 24h exposure to cisplatin. Instead, a decrease in levels of p21 after cisplatin treatment was observed. After 48 hours exposure to cisplatin, p21 was also transactivated in the three cell lines (data not shown), which is indicative of a delayed effect. On the other hand, p21 decreased after exposure to both cisplatin and DACH-acetato-Pt in cell lines with upregulation of basal p21 in functionally-negative p53 group. These observations did not change when the exposure time was extended to 48h (data not shown).

Time course change in G1 cell cycle regulators in ZR75-1 and T47D exposed to the platinum agents. In order to assess in detail the change in the levels of p21 and cell cycle-related proteins, the cisplatin-resistant wild-type p53 ZR75-1 and mutant p53 T47D cell lines were treated with 10μM of each drug for various lengthes of time and protein extacts were subjected to Western immunoblot analysis (Figure5). Time-dependent induction of p53 was observed in ZR75-1 cells exposed to each drug. However, p53 was induced more rapidly in cells exposed to DACH-acetato-Pt than in cells exposed to cisplatin. The rapid transactivation of p21 was evident with DACH-acetato-Pt, whereas, in contrast, a time-dependent decrease in levels of p21 was seen with cisplatin. Decrease in p21 was also observed with time in the T47D cell line exposed to each drug. Interestingly, expression of Cyclin D1 was decreased in parallel with p21 in both cell lines, although no significant change was observed in levels of Cyclin D1 in ZR75-1 treated with DACH-acetato-Pt. There were no gross changes in Cyclin E and p27 in both cell lines treated with each drug. Thus, the expression of the five genes was not consistent with the greater cytotoxicity of DACH-acetato-Pt relative to cisplatin in both cell lines.

Dose-response change in the levels of p21 and Cyclin D1 and G1/S progression of cell cycle. To investigate if the changes in p21 and Cyclin D1 levels affect the cell cycle, ZR75-1 and T47D cells were treated with a range of concentrations of cisplatin or DACH-acetato-Pt, and the expressed levels of the proteins and cell cycle phase distributions were analyzed. Decrease in the levels of p21 was evident in ZR75-1 cells exposed to lower concentrations of cisplatin, whereas the levels of p21 was increased at lower concentrations of DACH-acetato-Pt, but decreased at higher concentrations (Figure 6A). The levels of Cyclin D1, on the other hand, were decreased in ZR75-1 cells exposed to cisplatin in a concentration-dependent manner, while higher concentrations of DACH-acetato-Pt were needed for the decrease in levels of Cyclin D1. In mutant p53 T47D cell line, the nadir of p21 expression was seen with 10 μM cisplatin and 20 μM DACH-acetato-Pt (Figure 6B). The difference may be due to the relative levels of platinum-DNA adducts formed. Similarly, a decrease in the levels of Cyclin D1 was severe even at the lowest concentration of cisplatin, while the effect of DACH-acetato-Pt was relatively mild. Cell cycle analysis revealed that the changes in p21 expression correlated with the changes in S-phase fraction (SPF) of the cell cycle. The correlation was more significant in T47D cell line with mutant p53 than in ZR75-1 cell line with wild-type p53. In T47D cells, the decrease in p21 expression showed an inverse pattern to the increase in SPF (Figure 7A-C). In the ZR75-1 cell line, the inverse relationship between p21 levels and changes in SPF is also observed, except high concentration ranges of DACH-acetato-Pt at 48h time point (Figure 7D-F). High concentrations of DACH-acetato-Pt arrested ZR75-1 cells in G1-phase, although the significant decrease in p21 was observed with 24h exposure.

Effects of ubiquitin-proteasome inhibitor on the decrease in p21 and Cyclin D1. In order to examine the possible role of ubiquitin-proteasome degradation pathway for the decrease in p21 and Cyclin D1 proteins, ZR75-1 and T47D cell lines were pretreated with 20 μM N-Acetyl-Leu-Leu-Norleu-al (LLnL; a 26S proteasome inhibitor) following exposure to low (10 μM) or high (50μM) concentration of cisplatin or DACH-acetato-Pt for 16 hours. A low concentration of cisplatin and a high concentration of DACH-acetato-Pt without pretreatment of LLnL induced significant decrease of p21. However, the levels of p21 expression in LLnL-pretreated cells were similar between control and platinum-treated cells, suggesting that the alterations in p21 was related to the ubiquitin-proteosome pathway. On the other hand, the decrease in Cyclin D1 was not affected by LLnL. These results indicate that different mechanisms are involved in the decrease in Cyclin D1 and p21.

DISCUSSION

p21 is a major negative regulator of the G1 checkpoint by binding to and inhibiting the activities of most cyclin/CDK complexes {Sherr & Roberts 1999 54 /id}. Basal level of p21 is reported to be upregulated in some of breast cancer cell lines and tumor samples, in related to some genetic backgrounds. One of such factors is the status of estrogen receptor (ER). Reed et al.{Reed, Florems, et al. 1999 55 /id} investigated 77 node-negative breast cancer samples with immunohistochemistry and revealed that p21 overexpression was associated with positive ER status. Chen et al.{Chen, Lowe, et al. 1999 49 /id} examined 9 breast cancer cell lines and 60 breast tumor samples by Western blot analysis to detect p21, p27 and ER, and found that a strong association between the levels of p21 and ER in both cell lines and tumor samples. On the other hand, both HER2/neu signaling {Yu, Jing, et al. 1998 26 /id} and EGFR signaling {Bromberg, Fan, et al. 1998 57 /id}are reported to increase p21 protein levels. In our panel of breast cancer cell line, there were four cell lines with upregulation of basal p21, two of which were ER-positive with moderate overexpression of HER2/neu (ZR75-1 and T47D){Spink, Spink, et al. 1998 56 /id}, the MDA-MB-330 cell line demonstrated signifiacnt overexpression of both HER2/neu (this study) and EGFR (information from ATCC) and the MDA-MB-157 cell line had none of them. On the other hand, the ER-positive MCF-7 cell line and the SK-Br3 cell line with significant overexpression of HER2/neu demonstrated no evident overexpression of p21, suggesting that cause of the p21 upregulation in breast cancer cells was multifactorial.

In this study, we have investigated the sensitivity to cisplatin and DACH-acetato-Pt in the panel of breast cancer cell lines, and revealed that the basal levels of p21 correlated with the resistance to cisplatin. The effect of increased basal p21 level in tumor cells on the sensitivity to cisplatin is still controversial. For instance, Lincet et al.{Lincet, Poulain, et al. 2000 32 /id} reported that p21 gene transfer into SK-OV-3 and OVCAR3 ovarian cancer cell lines enhanced susceptibility to cisplatin-induced apoptosis. In contrast, several groups have reported the opposite results. Fan et al. {Fan, Chang, et al. 1997 15 /id} has reported that HCT-116 colon cancer cell line and murine embrionic fibroblast with disrupted p21 genes demonstrated significant sensitivity to cisplatin. They have also presented the reduced DNA repair activites in p21-disrupted cell lines. Ruan et al. {Ruan, Okcu, et al. 1999 18 /id} introduced antisense p21 adenovirus expression vector into glioma cell lines, and demonstrated that attenuation of p21 increased sensitivity to BCNU and cisplatin.

On the other hand, the cytotoxicity of DACH-acetato-Pt in breast cancer cell lines was dependent on the presence of functional p53, as we have previously reported with the ovarian cancer panel {Hagopian, Mills, et al. 1999 45 /id}. This compound was very efficient in inducing p53 and p21 in cisplatin-resistant ovarian tumor models {Hagopian, Mills, et al. 1999 45 /id}. In this study, DACH-acetato-Pt has also demonstrated its activity in inducing p21 in cell lines with wild-type p53 function. In contrast, it was surprising results that p21 was significantly decreased in all four cell lines with basal upregulation of p21 following exposure to cisplatin. Similar response in p21 was also observed with DACH-acetato-Pt in three cell lines lacking functional p53 with upregulation of basal p21. These results are the first evidence that p21 is down-regulated with exposure to platinum agents. Wang et al. {Wang, Fan, et al. 1999 48 /id} have reported that ultrviolet (UV) radiation down-regulates p21 expression in a variety of human cancer cell lines independently of p53 status, while ionizing radiation (IR) caused up-regulation of p21 in cells with wild-type p53 and little or no change in p21 in cells with mutant p53. On the other hand, Park et al. {Park, Carter, et al. 1999 1 /id} reported that IR also initiate negative signals toward the p21 promotor. These results suggest that cisplatin and UV activate similar signaling pathways that are distinct from those activated by DACH-acetato-Pt and IR, as we have mentioned previously {Hagopian, Mills, et al. 1999 45 /id} {Siddik, Mims, et al. 1998 52 /id}.

Western immunoblot analysis of the time-course treatment of the ZR75-1 and T47D cell lines with each drug have revealed that the expression levels of cyclin D1 were also decreased in parallel with p21. As both p21 and cyclin D1 are major cell cycle regulators, especially in G1/S checkpoint, the down-regulation of these protein molecules in response to platinum agents might induce cell cycle progression. Therefore, we have investigated dose-dependent analysis on the changes in p21 and cell cycle distribution. As a result, we found inverted correlation between the relative expression of p21 and the changes in SPF, suggesting that the decrease in p21 may contribute G1/S transition. Recently, in addition, some kinds of DNA damage are reported to increase activity of checkpoint kinases Chk1 and Chk2, which inhibit the kinase activity of cdk2 through phosphorylation, resulting in S-phase arrests {Sampath & Plunkett 2001 58 /id}. In fact, cisplatin-induced DNA damage is reported to activate Chk2 {Damia, Filiberti, et al. 2001 59 /id} and induce S-phase arrest {Hagopian, Mills, et al. 1999 45 /id}. These findings suggest that not only increased G1/S transition but also inhibition of S-phase progression contribute to the accumulation of the cells into S-phase. A checkpoint response induced by cytotoxic agents can have both positive and negative effects on the induction of apoptosis {Sampath & Plunkett 2001 58 /id}{Arooz, Yam, et al. 2000 2 /id}, and the relationship between the S-phase accumulation and the induction of apoptosis is still unclear. However, the increase in SPF in response to platinum agents seems to affect the resistance in these cell lines.

Interestingly, concentration effects were observed in the decrease in p21 in response to either drug. Lower concentrations of cisplatin demonstrated strong effect on the decrease in p21 protein, whereas high concentrations of DACH-acetato-Pt decreased p21 significantly. The difference in the effects on p21 between the drugs may be due to the difference in the extent of DNA adducts. On the other hand, in wild-type p53 ZR75-1 cells, lower concentrations of DACH-acetato-Pt induced p21 more efficiently than the same concentrations of cisplatin through the rapid induction of p53. As a result, there observed a big difference between cisplatin and DACH-acetato-Pt in the levels of p21 of the ZR75-1 cells treated with lower concentrations of each drug. The difference in the expression of p21 may explain more than 10-fold difference in DNA damage tolerance to cisplatin and DACH-acetato-Pt in ZR75-1 cell line.

We have pretreated cells with a proteasome inhibitor LLnL prior to drug exposure and revealed that the decrease in p21 was, in some extent, due to proteasomal degradation. However, LLnL failed to inhibit the decrease in cyclin D1, suggesting that there are different mechanisms involved in the decreased in p21 and cyclin D1. In addition, decrease in the levels of cyclinD1 was never observed in ZR75-1 cells treated with lower concentrations of DACH-acetato-Pt, where the p21 levels were higher than the basal level, as shown in Figure 5A and 6A. These results suggest that the decrease in cyclin D1 may be a secondary event following the down-regulation of p21.

In conclusion, the upregulation in basal levels of p21 in breast cancer cell lines correlated with cisplatin-resistance, while DACH-acetato-Pt was still effective against such tumor cells. On the other hand, the cytotoxicity of DACH-acetato-Pt was dependent on the presence of functional p53. These results suggest that DACH-acetato-Pt may have clinical potential against breast tumors, especially with wild-type p53 background.

Table 1. p53 status of breast cancer cell lines

Cell line	p53 gene status	DNA sequence	amino acid	References
MCF-7	wild-type			1
ZR75-1	wild-type			This study
HCC1937	mutant	306: CGA to TGA	Arg to Term	2
MDA-MB-330	mutant	220: TAT to TGT	Tyr to Cys	This study
MDA-MB-435	mutant	266: GGA to GAA	Gly to Glu	3
SK-Br3	mutant	175: CGC to CAC	Arg to His	4
T47D	mutant	194: CTT to TTT	Leu to Phe	~~5~~ 3
MDA-MB-157	null			~~6~~ 5
MDA-MB-436	null			~~6~~ 5

Table 2. IC$_{50}$ of cisplatin and DACH-acetato-Pt in breast cancer cell lines following continuous drug exposure

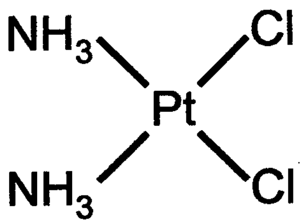
Cell line	IC$_{50}$ value (μM)		Cisplatin/DACH-acetato-Pt ratio
	Cisplatin	DACH-acetato-Pt	
functional p53 (+)			
MCF-7	0.83 ± 0.27*	0.17 ± 0.12	4.88
ZR75-1	9.58 ± 1.59	0.85 ± 0.29	11.27
HCC1937	0.65 ± 0.11	0.23 ± 0.05	2.83
functional p53 (-)			
MDA-MB-330	7.43 ± 2.55	1.10 ± 0.44	6.75
MDA-MB-435	0.84 ± 0.06	1.11 ± 0.36	0.76
SK-Br3	0.59 ± 0.24	1.38 ± 0.09	0.43
T47D	4.89 ± 0.30	1.84 ± 0.52	2.66
MDA-MB-157	0.75 ± 0.23	1.29 ± 0.49	0.58
MDA-MB-436	0.16 ± 0.12	0.25 ± 0.14	0.64

* Mean ± SD; n=3-5

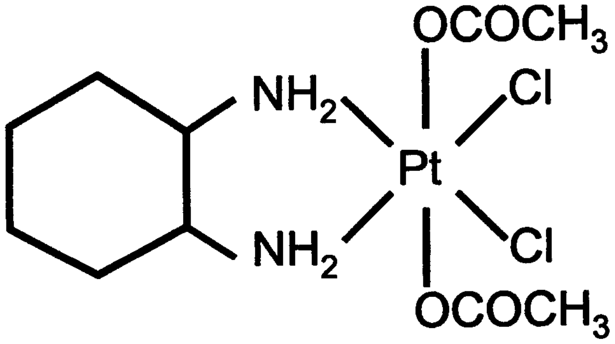
Table 3. Biochemical pharmacology of cisplatin and DACH-acetato-Pt against ZR75-1 and T47D breast cancer cell lines

Cell Line	ZR75-1		T47D	
Drugs	cisplatin	DACH-acetato-Pt	cisplatin	DACH-acetato-Pt
2-h platinum uptake (ng Pt/mg protein)	71.4 ± 5.3*	58.0 ± 13.5	59.8 ± 9.4	63.3 ± 8.6
DNA adducts at 2-h (ng Pt/mg DNA)	30.6 ± 7.1	20.4 ± 2.6	22.7 ± 3.0	9.3 ± 0.7
DNA damage tolerance (ng Pt/mg DNA at IC50)	2.9 ± 0.7	0.17 ± 0.02	1.1 ± 0.2	0.17 ± 0.01

* Mean ± SD



Cisplatin



1R,2R-DACH-$(Ac)_2Cl_2$-Pt(IV)

Figure 1

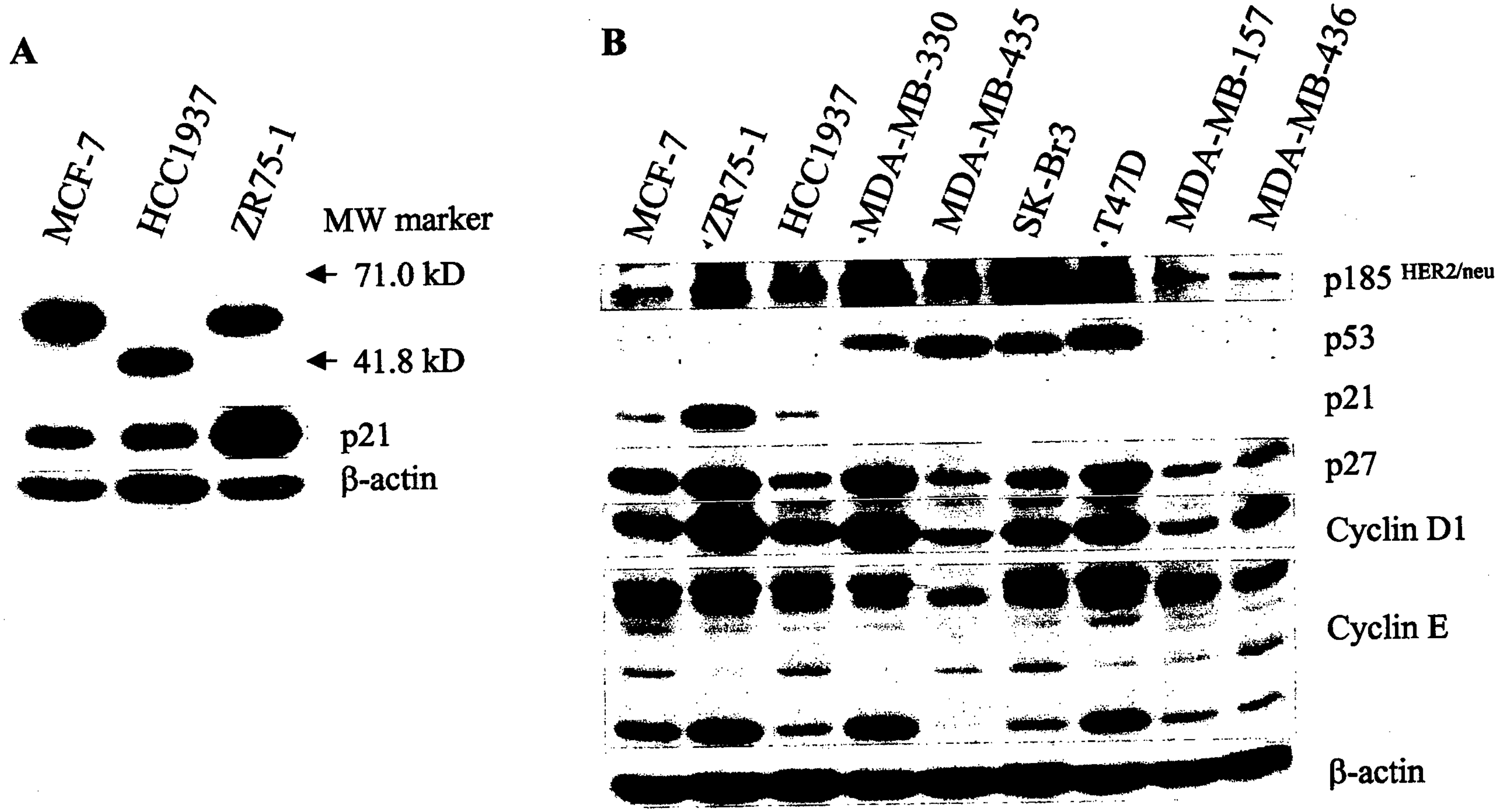

A
MCF-7
HCC1937
ZR75-1
MW marker
71.0 kD
41.8 kD
p21
β-actin
B
MCF-7
ZR75-1
HCC1937
MDA-MB-330
MDA-MB-435
SK-Br3
T47D
MDA-MB-157
MDA-MB-436
p185 HER2/neu
p53
p21
p27
Cyclin D1
Cyclin E
β-actin

Figure 2

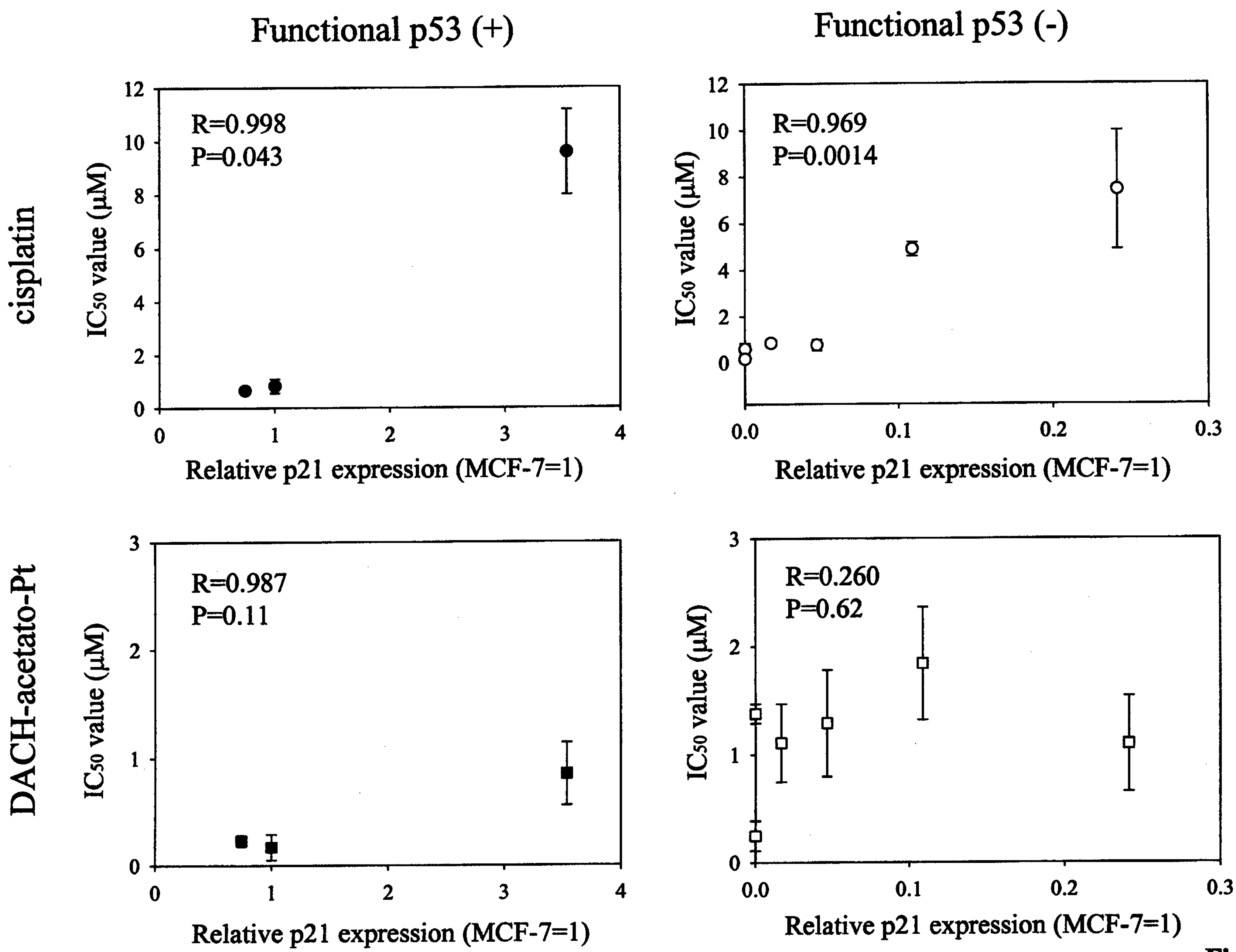

Functional p53 (+)
Functional p53 (-)
cisplatin
DACH-acetato-Pt
R=0.998
P=0.043
R=0.969
P=0.0014
R=0.987
P=0.11
R=0.260
P=0.62
IC50 value (µM)
Relative p21 expression (MCF-7=1)
0
2
4
6
8
10
12
0
1
2
3
4
0.0
0.1
0.2
0.3

Figure 3

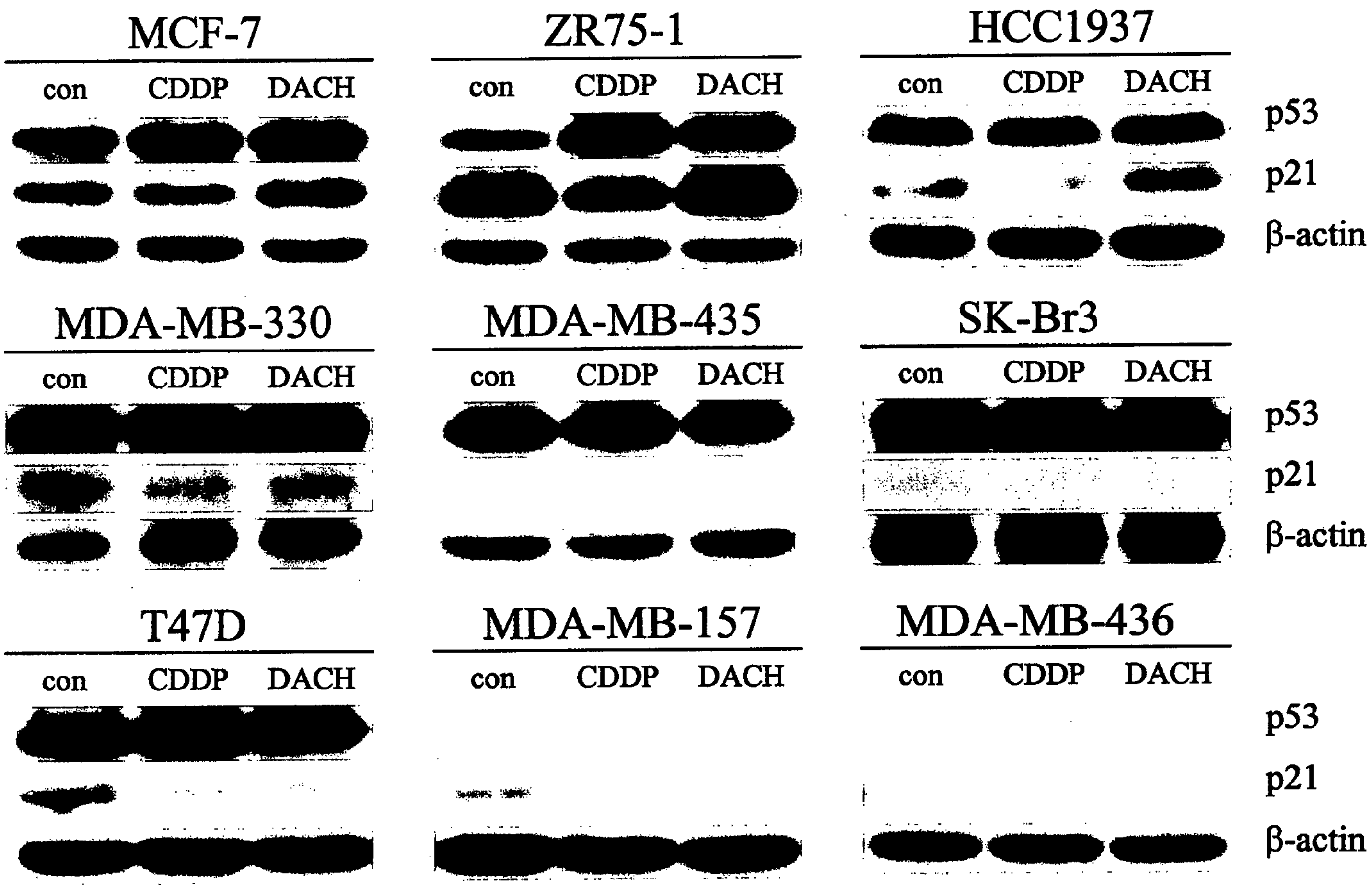

MCF-7
con
CDDP
DACH
ZR75-1
con
CDDP
DACH
HCC1937
con
CDDP
DACH
p53
p21
β-actin
MDA-MB-330
con
CDDP
DACH
MDA-MB-435
con
CDDP
DACH
SK-Br3
con
CDDP
DACH
p53
p21
β-actin
T47D
con
CDDP
DACH
MDA-MB-157
con
CDDP
DACH
MDA-MB-436
con
CDDP
DACH
p53
p21
β-actin

Figure 4

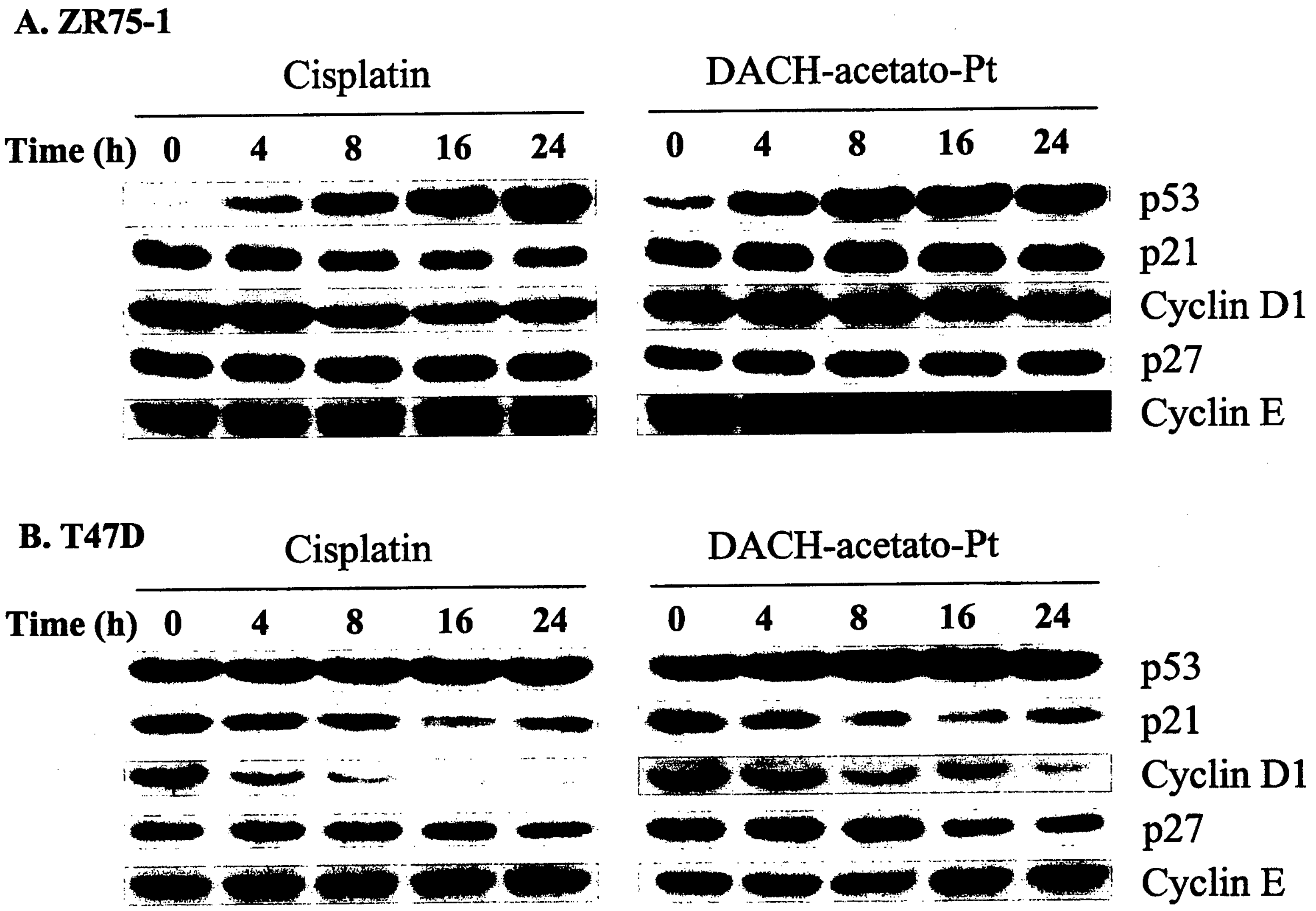
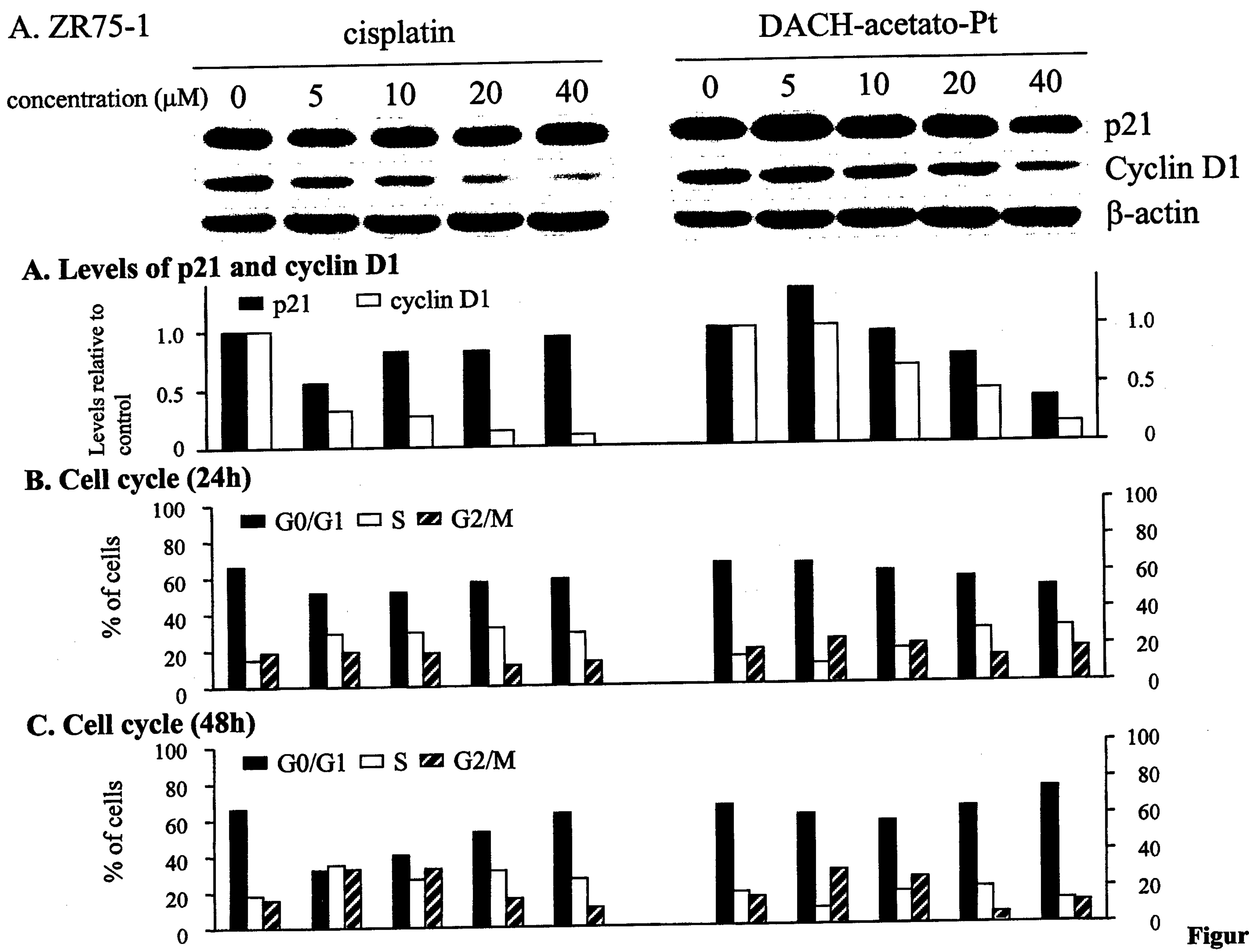

A. ZR75-1
Cisplatin
DACH-acetato-Pt
Time (h) 0 4 8 16 24
0 4 8 16 24
p53
p21
Cyclin D1
p27
Cyclin E
B. T47D
Cisplatin
DACH-acetato-Pt
Time (h) 0 4 8 16 24
0 4 8 16 24
p53
p21
Cyclin D1
p27
Cyclin E

Figure 5



A. ZR75-1

A. Levels of p21 and cyclin D1

B. Cell cycle (24h)

C. Cell cycle (48h)

Figure 6A

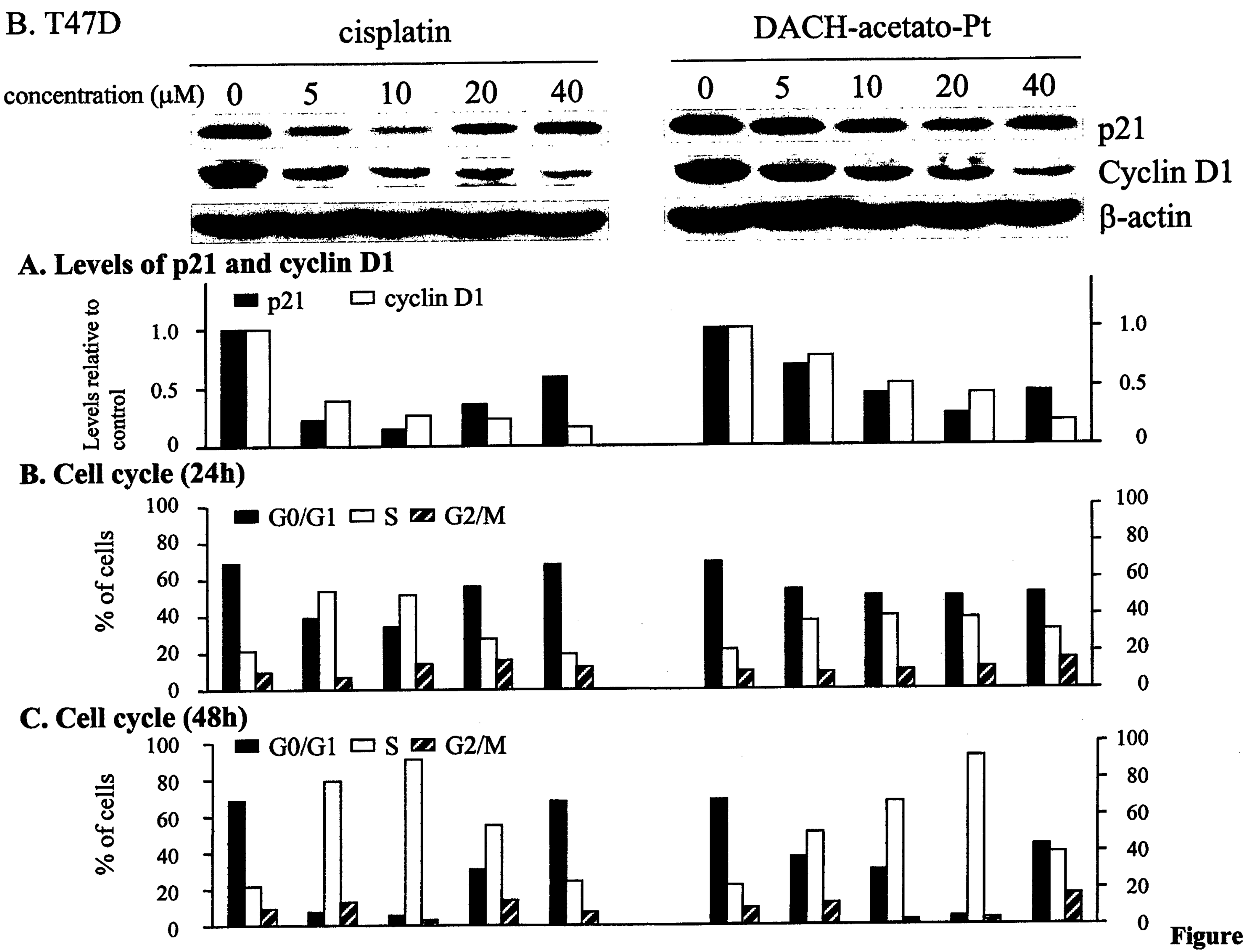

B. T47D
cisplatin
DACH-acetato-Pt
concentration (μM) 0 5 10 20 40
0 5 10 20 40
p21
Cyclin D1
β-actin
A. Levels of p21 and cyclin D1
p21
cyclin D1
Levels relative to control
1.0
0.5
0
B. Cell cycle (24h)
G0/G1
S
G2/M
% of cells
100
80
60
40
20
0
C. Cell cycle (48h)
G0/G1
S
G2/M
% of cells
100
80
60
40
20
0

Figure 6B

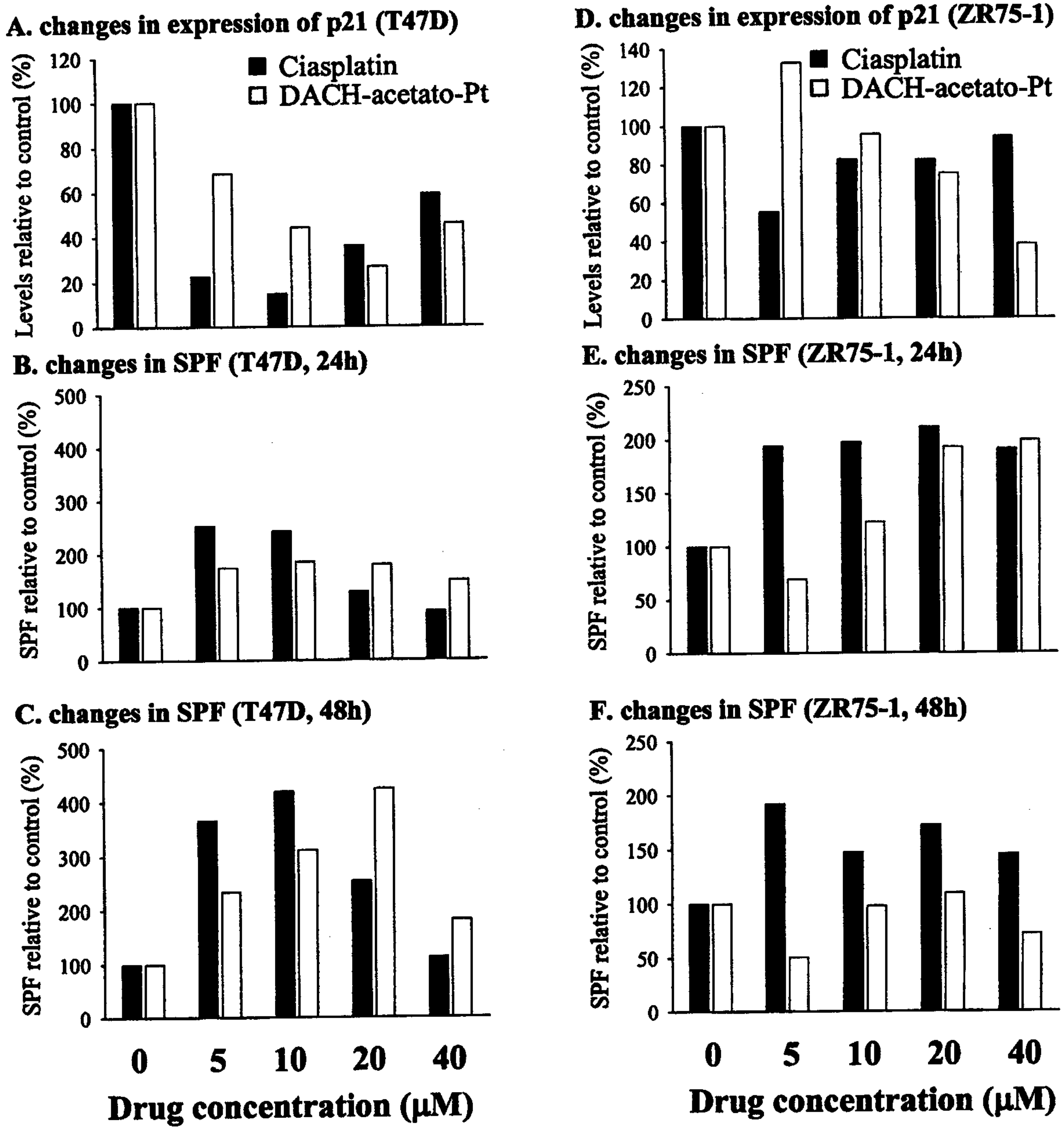

A. changes in expression of p21 (T47D)
Levels relative to control (%)
120
100
80
60
40
20
0
■ Ciasplatin
□ DACH-acetato-Pt
B. changes in SPF (T47D, 24h)
SPF relative to control (%)
500
400
300
200
100
0
C. changes in SPF (T47D, 48h)
SPF relative to control (%)
500
400
300
200
100
0
0
5
10
20
40
Drug concentration (μM)
D. changes in expression of p21 (ZR75-1)
Levels relative to control (%)
140
120
100
80
60
40
20
0
■ Ciasplatin
□ DACH-acetato-Pt
E. changes in SPF (ZR75-1, 24h)
SPF relative to control (%)
250
200
150
100
50
0
F. changes in SPF (ZR75-1, 48h)
SPF relative to control (%)
250
200
150
100
50
0
0
5
10
20
40
Drug concentration (μM)

Figure 7

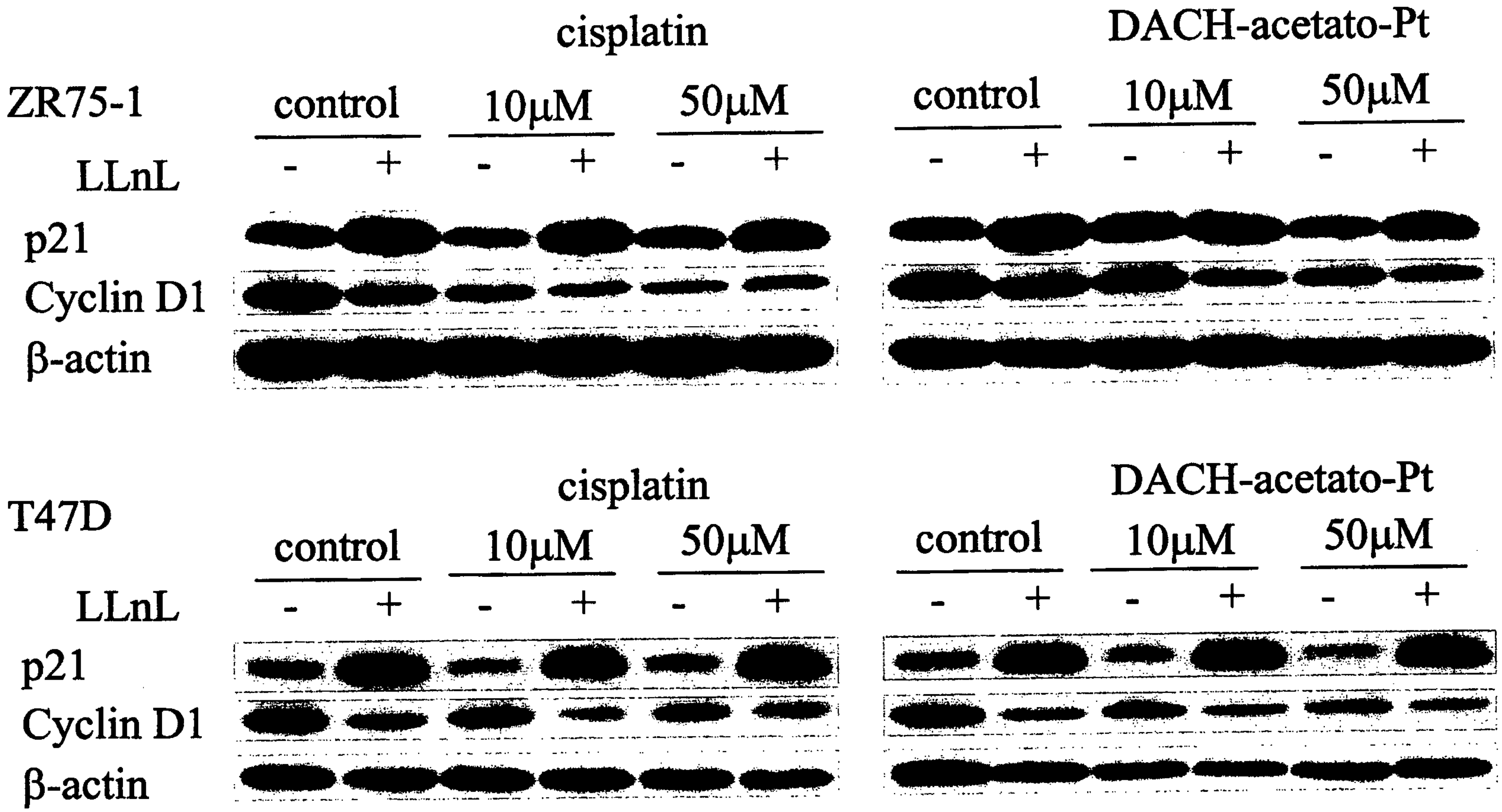

cisplatin
DACH-acetato-Pt
ZR75-1
control
10μM
50μM
control
10μM
50μM
LLnL
- + - + - +
- + - + - +
p21
Cyclin D1
β-actin
cisplatin
DACH-acetato-Pt
T47D
control
10μM
50μM
control
10μM
50μM
LLnL
- + - + - +
- + - + - +
p21
Cyclin D1
β-actin

Figure 8

Wild-type p53 reduces DNA-damage tolerance of HER2/neu-overexpressing ovarian cancer cells in a drug-dependent manner

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Running Title: p53 and platinum against HER2-overexpressing tumor

Key words: HER2/neu, p53, platinum, ovarian cancer, DNA damage tolerance.

FOOTNOTES

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[3] The abbreviations used are: EGFR, epidermal growth factor receptor; DACH-acetato-Pt, 1R,2R-diaminocyclohexan-diacetato-dichloro-platinum (IV); FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide.

ABSTRACT

In order to evaluate the effects of p53 on the cytotoxicity of cisplatin and novel analog DACH-acetato-Pt against HER2/neu-overexpressing tumor cells, we established clones by transfecting the null-p53, HER2/neu-overexpressing SK-OV-3 ovarian tumor model with a temperature-sensitive mutant (TS) p53. At 37 °C when TS-p53 functioned as a mutant, drug sensitivity of the TS9 clone with low p53 expression was unaffected, whereas the TS4 clone with high p53 expression demonstrated up to 2-fold resistance to cisplatin (IC_{50}: 0.84 vs. 1.63 μM), suggesting that the mutant p53 has gain-of-function on cell survival. At 32 °C when TS-p53 functioned as a wild-type, sensitivity to cisplatin was comparable between TS and neo, but the sensitivity to DACH-acetato-Pt was increased 2- to 5-fold in TS clones (IC_{50}: 14.3 vs. 5.91 μM in clone 4, IC_{50}: 22.7 vs. 4.63 in clone 9). Modulation of cytotoxicity of TS4 cells was not due to changes in intracellular drug uptake or the formation of DNA adducts. However, the data indicated that functional p53 either normalized or decreased tolerance to DNA damage induced by the platinum agents. Western analysis demonstrated that cisplatin-induced DNA damage significantly activated the Akt pathway through increased phosphorylation of $p185^{HER2/neu}$, whereas the effect of DACH-acetato-Pt on $p185^{HER2/neu}$ phosphorylation was transient and limited. Although at 32 °C p53 was induced to a similar extent by each drug, p21 was transactivated more efficiently by DACH-acetato-Pt than by an equitoxic concentration of cisplatin in TS4. These results suggest that the inhibition of wild-type p53 function is associated with increased levels of phosphorylated $p185^{HER2/neu}$. Our studies provide evidence that activation of functional p53 not only normalizes sensitivity of cells overexpressing HER2/neu to cisplatin by circumventing the dominant-negative

effects of mutant p53, but also increases sensitivity to DACH-acetato-Pt by over-riding the effects of HER2/neu overexpression. Thus, a combination of wild-type p53 and DACH-acetato-Pt may be an effective treatment against tumors overexpressing HER2/neu.

INTRODUCTION

HER2/neu encodes a 185 kD transmembrane glycoprotein which is a member of the type I receptor tyrosine kinase family with homology to EGFR[3] (1). This gene is often amplified and/or overexpressed in a variety of human tumors (2,3), indicating its critical role in the development of human cancers. In breast and ovarian cancers, for instance, the incidence of HER2/neu amplification is reported to be 10-34 % (4) and 19-59 % (5), respectively. Amplification/overexpression of this oncogene is also reported to correlate with poor prognosis, as it enhances the metastatic potential of cancer cells (6) and induces resistance to various chemotherapeutic agents (7,8).

The antitumor agent cisplatin is highly effective in the clinic against human cancers (9,10). However, the presence of primary or the emergence of secondary resistance significantly undermines the clinical utility of this drug (11). Several reports indicate that amplification/overexpression of HER2/neu can directly reduce cisplatin cytotoxicity (12,13). Considering that tumor cells rarely have a single genetic defect, it needs to be recognized that the presence of other genetic abnormalities may also contribute to the overall poor prognosis. One such abnormality relates to the p53 gene. In fact, HER2/neu amplification was found in 41% of the samples with p53 abnormalities as compared to 15.9% of the samples without p53 abnormalities (14). The tumor suppressor p53 is mutated or deleted in over 50% of all human tumors (15). As 'guardian of genome', wild-type p53 mediates cell-cycle arrest or apoptosis in response to DNA damage (16), and loss of p53 function is associated with resistance to apoptosis induced by chemotherapy and radiotherapy (17,18). Recently, several preclinical studies suggest that transfer of wild-type p53 combined with conventional doses of radiation or chemotherapeutic agents may have a synergistic effect without additional toxicity (19,20).

Recent studies suggest that there may be a link between signal transduction pathways for HER2/neu and p53 (21,22). Casalini et al. (22), for instance, have demonstrated that HER2/neu overexpression led to tumor cell proliferation in the IGROV1 /Pt1 model with mutant p53, but resulted in apoptosis in IGROV1 cells harboring wild-type p53. Similarly, the ras-mediated signal transduction pathway, which is a downstream target of HER2/neu signal (23), inhibits p53 function (24) and is associated with resistance to therapeutic DNA damaging agents (25). On the other hand, Zhou et al. (26) reported that overexpression of HER2/neu induced MDM2 phosphorylation through the phosphatidylinositol-3'-OH kinase (PI3K)- Akt pathways and the phosphorylated MDM2 increased p53 ubiquitination. We have demonstrated that the DNA damaging antitumor agent cisplatin and the non-cross-resistant analog 1R,2R-diaminocyclohexane-diacetato-dichloro-platinum (IV) (DACH-acetato-Pt; Figure 1), were less cytotoxic against the ovarian tumor SK-OV-3 model (27), which overexpresses HER2/neu and is null for p53 (28). However, the analog was very effective against cisplatin-resistant tumor cell lines harboring wild-type p53 (27). Therefore, it is possible that the lack of effectiveness of DACH-acetato-Pt against SK-OV-3 cells may be due to the absence of p53.

Based on the above information, we hypothesized that introduction of functional p53 in combination with DACH-acetato-Pt could be an effective treatment against tumors overexpressing HER2/neu. In order to test the hypothesis, we transfected SK-OV-3 with a temperature-sensitive (TS) p53 vector which assumes mutant p53 configuration at 37

°C and wild-type at 32 °C (29). We report here that the cytotoxicity of DACH-acetato-Pt was significantly enhanced in the presence of functional p53, although it did not alter the impediment of HER2/neu overexpression on cisplatin-mediated cytotoxicity.

MATERIALS AND METHODS

Chemicals. Cispaltin was obtained from Sigma Chemical Co. (St.Louis, MO). We have previously reported the synthesis and chemical characterization of DACH-acetato-Pt (30). Cisplatin and DACH-acetato-Pt were dissolved in normal saline and water, respectively, then sterilized through 0.22-μm disc filter. The concentration of each drug was confirmed by flameless atomic absorption spectroscopy (FAAS) (31). MTT was purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Culture and Transfection. SK-OV-3, which was originally established from malignant ascites of a patient treated with thiotepa (32), was obtained from American Type Culture Collection (Rockville, MD), and maintained in McCoy's 5A supplemented with 10% heat-inactivated FBS, 2 mM L-glutamin, and antibiotics under a humidified atmosphere of 5% CO_2. SK-OV-3 lacks expression of p53 mRNA and protein, and overexpresses HER2/neu (28). pC53NN M133T, which is a plasmid containing a TS-p53 cDNA under the control of a CMV promotor was kindly provided by Dr. Guillermina Lozano (The University of Texas, M.D. Anderson Cancer Center, Houston, TX). The plasmid was introduced into cells using FuGENE 6 transfection reagent. After 14 days culture in the selective medium containing 600 μg/ml G418, G418-resistant colonies were selected and expanded. The clones were examined for their stable growth characteristics and expression of p53. Two clones with different levels of p53 expression (TS4 and TS9) were used in this study. Two G418-resistant sub-clones derived from TS4 and TS9 that no longer expressed p53 were used as controls (neo4 and neo9, respectively). Cells were maintained in McCoy's 5A supplemented with 10% heat-inactivated FBS, 2 mM L-glutamin, and 600 μg/ml G418, and examined regularly for p53 expression.

Cytotoxicity and Biochemical PharmacologyStudies. Cells were seeded in 96-well plates, allowed to attach for 24 hours and then either maintained for another 24 hours at 37 °C or shifted to 32 °C. Following acclimation, cells were exposed to various concentrations of cisplatin or DACH-acetato-Pt. After 5 days of incubation at 37 °C or 7 days at 32 °C, the sensitivities of the cells to the platinum agents were evaluated using a modified MTT assay (33). Evaluations in attached cells of cellular platinum uptake and DNA adduct formation were conducted as described previously (34,31). Briefly, cells treated with 100μM of cisplatin or DACH-acetato-Pt for 2 hours at each temperature were collected and washed. For determination of cellular uptake, cell pellets were first digested overnight at 55 °C in 50 μl of 1 M hyamine hydroxide (ICN, Irvine, CA). To measure platinum-DNA adduct formation, high molecular weight DNA was isolated from cell pellets according to standard procedures (35). The platinum content of both samples was determined by FAAS. Platinum-DNA damage tolerance, defined as the level of DNA adducts at IC50, was assessed as previously reported (36).

Western Analysis Cells were acclimated at 32 °C for 24 hours prior to drug exposure, and were exposed to 5 x IC_{50} concentrations of cisplatin or DACH-acetato-Pt (29.3 μM cisplatin or 29.6 μM DACH-acetato-Pt) for 48 hours. At 12-hour intervals, cells were collected, washed twice with ice-cold phosphate-buffered saline and lysed for 20 min on ice with 100 μl of lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 100 μg/ml phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin). The lysates were collected by microcentrifugation at 4 °C, and then the protein was determined by the standard Lowry procedure. Forty μg of total cell protein was electrophoresed on a 10% SDS-polyacrylamide gel, transferred onto nitrocellulose membranes, and incubated with various antibodies. Mouse monoclonal anti-p53 (Ab-6) was obtained from Oncogene Research Products (Cambridge, MA), and anti-p21 (Cip1/Waf1) antibody was purchased from Transduction Laboratories (Lexington, KY). Rabbit polyclonal anti-phospho-ErbB2 (Y1248) was obtained from upstate biotechnology (Lake Placid, NY). Rabbit polyclonal anti-phospho-p53 antibodies (serine 15 and serine 392), anti-phospho-Akt (serine 473) and anti-phospho-Erk1/Erk2 MAPK (Thr202/Tyr 204) were purchased from Cell Signaling Technology (Beverly, MA). Mouse monoclonal anti-β-actin antibody was purchased from Sigma (St. Louis, MO). All immunoblots were visualized by enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL), and quantified by laser densitometry.

Statistical Analysis. Differences between groups were evaluated by Student's *t*-test.

RESULTS

Establishment of Stable Transfectants. We utilized two TS clones with different levels of p53 expression and the corresponding neo clones, which were used as controls (Figure 2A). All clones showed stable growth, even at 32 °C when p53 functions as wild-type. The expression of p53 in TS4 at 37 °C was almost 3-fold higher than that in TS9, while both neo clones did not express p53. HER2/neu was overexpressed in all clones to the same extent, and the levels of the expression were almost 50-fold higher than that in MCF-7 breast cancer cell line, which was used as a negative control. Basal levels of p53 and expression of p53-dependent transactivation of p21 in TS and neo clones under different temperature conditions are shown in Figure 2B. Expression of p21 was apparent in both of TS clones only at 32 °C, and this was consistent with the understanding that the introduced p53 functioned as wild-type at 32 °C and was able to transactivate the p21 (Wafl/Cip1) gene. The extent of p21 expression was almost 5-fold higher in TS4 at 32 °C than that in TS9.

Cytotoxicity of Cisplatin and DACH-acetato-Pt. The results of cytotoxic evaluation are shown in Table 1. TS4 demonstrated about 2-fold resistance to cisplatin compared to neo4 at 37 °C (IC_{50}: 0.84 vs. 1.63 μM) whereas resistance to DACH-acetato-Pt was not significant (1.58 vs. 2.19 μM). However, there was no significant difference between neo9 and TS9 in the sensitivity to both drugs at 37 °C. These results suggest that high expression levels of mutant p53 increased significant resistance to cisplatin only, while low levels of mutant p53 did not affect the cytotoxicity of the drugs. At 32 °C, on the other hand, there was no significant difference in the cytotoxicity of cisplatin between neo and TS in both clone 4 and clone 9. In contrast, the sensitivity to DACH-acetato-Pt was significantly increased in both TS4 and TS9 compared to each neo clone (IC_{50}: 14.3 vs. 5.91 μM in clone 4, IC_{50}: 22.7 vs. 4.63 in clone 9), and TS/neo IC_{50} ratio, as an index of resistance, was decreased to 0.41 and 0.20 in clone 4 and 9, respectively. These results indicate that functional p53 increases sensitivity of HER2/neu overexpressing SK-OV-3 cells to DACH-acetato-Pt, although it only normalized or retained the cisplatin cytotoxicity to that seen in control cells.

Biochemical Pharmacology of Cisplatin and DACH-acetato-Pt. In order to rationalize the cytotoxicity data, the biochemical pharmacology of the platinum agents was investigated in neo4 and TS4 at each temperature (Table 2). Cellular uptake of platinum was similar in both cell lines at either temperature. Furthermore, there was no significant difference in DNA adducts between the cell lines. However, at 37 °C, DNA damage tolerance to cisplatin was significantly increased in the TS4 clone compared to neo. In contrast, there was no difference in damage tolerance to cisplatin at 32 °C. On the other hand, DNA damage tolerance to DACH-acetato-Pt was significantly decreased in TS4 compared to neo4 at 32 °C (1.13 vs. 0.51 ng Pt/mg DNA). These results suggest that changes in sensitivity to cisplatin and DACH-acetato-Pt by alterations in functional p53 status correlates with inverse changes in the tolerance to platinum adducts.

Effects of drug-induced DNA damage on HER2/neu signaling and the activation of wild-type p53. In order to understand the differential modulation by wild-type p53 on the cytotoxicity of cisplatin and DACH-acetato-Pt, studies were undertaken to investigate the effect of drug-induced DNA damage on the activation of HER2/neu signaling and activation of p53: TS4 cells were exposed to equitoxic concentrations of each drug at 32 °C and cell extracts were subjected for Western immunoblot analysis. The activity of HER2/neu signaling was evaluated by phosphorylation status of $p185^{HER2/neu}$ and two major down-stream targets Akt and Erk1/2. On the other hand, the function of p53 is regulated by phosphorylation at several sites on the p53 molecule, including serine 15 and serine 392 (16). Serine 15 phosphrylation appears to be closely associated with induction of p53 through an increase in protein stabilization (16). Therefore, in order to assess the function of introduced p53 protein in response to the platinum agents, the phosphorylation status of p53 and transactivation of p21 were examined. The Western immunoblots and results of densitometric analysis of the immunoblots are shown in Figure 3 and 4, respectively. Although no change in levels of total HER2/neu expression was observed during the time course (data not shown), the active, phosphorylated form of $p185^{HER2/neu}$ was significantly increased and peaked in TS4 cells treated with cisplatin, whereas the increase of phospho-$p185^{HER2/neu}$ in cells exposed to DACH-acetato-Pt was relatively low with a peak effect observed at 12 h (Figure 4A). A significant time-dependent increase in phosphorylation of Akt and decrease in phosphorylation of Erk1/2 was observed in TS4 cells exposed to cisplatin, although a transient, low-level increase in phosphorylation of these proteins was seen with DACH-acetato-Pt (Figure 4B, C). These results inidcate that cisplatin-induced DNA damage activates HER2/neu signaling which induces the PI3K-Akt pathway, whereas DNA damage induced by DACH-acetato-Pt has reduced effect on HER2/neu signaling. The levels of phospho-$p185^{HER2/neu}$ and phospho-Akt were not affected by temperature shift from 37 °C to 32 °C (data not shown). Total p53 was induced with time in TS4 cells exposed to both drugs (Figure 4D). Phosphorylation of p53 at both serine 15 and serine 392 was evident in cells treated with cisplatin, whereas lesser extent of serine 15 phosphorylation and no detectable levels of phosphorylation at serine 392 were observed in cells exposed to DACH-acetato-Pt. Although the induced levels of total p53 were similar in cells exposed to cisplatin and DACH-acetato-Pt treatment, the levels of p53 phosphorylated at serine 15 were grossly different (Figure 4E). Moreover, the increase in this phosphorylated form of p53 by cisplatin paralleled the increase in total p53, whereas the increase in phosphorylated p53 by DACH-acetato-Pt was observed much later. Changes in levels of total and phospho-p53 and p21 were not observed in either neo4 cells treated with either drug at 32 °C or in TS4 cells treated at 37 °C (data not shown). These results indicate that increase in p53 can also occur through a mechanism not involving serine 15 phosphorylation. Increase in the levels of p21 protein was also seen with time in cells treated with each drug (Figure 4F). These results suggest that the TS p53 acted as wild-type at 32 °C. However, the extent of p21 transactivation was 2-fold higher in cells treated with DACH-acetato-Pt than that with cisplatin. These results suggest that different pathways are involved in p53 activation with the two platinum agents, and that differential post-translational modifications of p53 may explain the different extent of p21 transactivation. It is clear that phosphorylation on neither serine 15 nor serine 392 paralleled the increase in p21 following exposure of cells to DACH-acetato-Pt.

DISCUSSION

Human ovarian SK-OV-3 tumor cells are null for p53 and overexpress HER2/neu (28), and our previous investigations have demonstrated that these molecular impediments cause the SK-OV-3 model to display resistance to cisplatin (11,27). The findings confirmed literature reports (12,13,37,38) and were consistent with the reported demonstration that tumor cells overexpressing HER2/neu can be sensitized to cisplatin and other antitumor agents by either down-regulating the HER2/neu pathway with an antibody approach (39,40) or restoring wild-type p53 by transfection (41). Results from our present studies are surprising in that they indicate that expression of mutant p53 in the already p53-deficient SK-OV-3 cells induced further resistance to the platinum agent. That mutant p53 was the factor responsible for the increased resistance was inferred from the observation that, at the permissive temperature of 32°C, when p53 functioned as wild-type, the increased resistance (that is, reduced cytotoxicity) was no longer apparent. In contrast, the cytotoxicity of the platinum analog DACH-acetato-Pt was not affected by mutant p53, but significantly increased by wild-type p53.

Our studies using the SK-OV-3 model were facilitated by establishing clones after transfecting tumor cells with a TS-p53 vector that carried a TS mutation at codon 133 (ATG to AGG; M133T). This mutation was initially identified in familial Li-Fraumeni breast sarcoma (42) and was later demonstrated to be temperature-sensitive (G. Lozano, unpublished observation). The two TS clones (TS4 and TS9) were selected for their differential level of expression of mutant p53 (at 37°C), which did not alter the basal expression of HER2/neu. Utilization of these clones in our investigations has demonstrated that there may be a threshold effect for mutant p53 to induce further resistance to cisplatin; only the clone expressing the higher level of mutant p53 was resistant to the parental drug. This increase in resistance can be reconciled by the understanding that mutant p53 can acquire gain-of-function (43), which promotes cell growth and interferes with apoptosis induced by antitumor agents, including cisplatin (44). Similarly, Blandino et al. (45) have reported that specific p53 mutants confer upon tumor cells a selective survival advantage during chemotherapy. It is reasonable to propose that in our studies the M133T-p53 at 37 °C has gain-of-function properties, which promote cell survival by increasing DNA damage tolerance and, thereby, reducing the cell killing effects of cisplatin. When the clones were influenced to express wild-type p53, the gain-of-function property was eliminated and cytotoxicity of cisplatin was restored to that of control cells. Thus, the wild-type p53 was unable to overcome the inherent cisplatin resistance due to HER2/neu overexpression. In this respect, the effect of increased levels of $p185^{HER2/neu}$ is dominant over wild-type p53 when cisplatin is the DNA-damaging agent. This was consistent with our present observation that restoring wild-type p53 function did not increase basal levels of apoptosis, as has been demonstrated to be the case in some tumor cell lines following transfection with wild-type p53 (46). It is interesting to note, however, that we were unable to generate stable clones following transfection of SK-OV-3 cells with a plasmid containing the full-length wild-type p53 cDNA (data not presented).

Although our data demonstrated that cisplatin resistance was loosely associated with the level of expression of mutant p53, it is possible that mutant p53 may have different effects in individual transfectant clone. This would be consistent with the controversy that exists over the role of wild-type p53 in drug sensitivity or resistance.

For instance, our previous studies have demonstrated that cisplatin resistance was substantially greater in ovarian tumor models harboring wild-type p53 than mutant or null p53 (27). Similarly, other reports have demonstrated that restoring (46,47) and inactivating (17, 48)wild-type p53 function can both enhance tumor cell sensitivity. Thus, there exists the likelihood that other factors influence cellular response to antitumor agents. A molecular factor that has the potential to modulate p53 function is overexpression and/or amplification of the HER2/neu oncogene (21,22). Indeed, recent reports suggest that HER2/neu signaling impacts wild-type p53 activity through both the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3'-OH kinase pathways (26,49). Our corresponding pilot studies with the wild-type p53 MCF-7 breast tumor model indicate that overexpression of HER2/neu reduced transactivation of p21 in response to cisplatin treatment (50). In the present study, the extent of p21 induction by cisplatin was also low and not consistent with the high degree of serine 15 phosphorylation, which usually contributes to activation of p53 function (16). This suggests that in the SK-OV-3 model, overexpression of HER2/neu inhibits p21 induction by down-regulating p53 function. These results are consistent with those reported by Casalini et al. (22), who demonstrated that although p53 expression was increased following HER2/neu transfection, MDM2 as the p53 transactivation target was also substantially down-modulated. Thus, the dominant effect of p185$^{HER2/neu}$ over p53 is likely related to inhibition of p53 function.

An important finding in our present study is that HER2/neu overexpression only affected p53 function when cisplatin was the inducer of wild-type p53. With the analog DACH-acetato-Pt, p53-mediated transactivation of p21 appeared to be efficient. This occurred even though serine 15 phosphorylation of p53 was relatively delayed and ser-392 phosphorylation was unaffected by the analog. The data suggest that the p53 activated by cisplatin is not only distinctly different from that activated by DACH-acetato-Pt, but also that its function is differentially affected by HER2/neu overexpression. This insight is novel and demonstrates that the functional p53, activated by a post-translational process that appears to be independent of serine 15 and serine 392 phosphorylation sites, is not inhibited by $p185^{HER2/neu}$ or its signaling pathway. Consequently, the presence of wild-type p53 sensitized TS clones to DACH-acetato-Pt by reducing the threshold of DNA damage tolerance. These findings with the platinum analog are in keeping with our previous observation that this agent can circumvent cisplatin resistance only in the presence of wild-type p53 (27).

An alternative factor that may contribute to the increased cytotoxicity of DACH-acetato-Pt but not that of cisplatin at 32°C may relate to their comparative effects on the $p185^{HER2/neu}$ pathway, as demonstrated by the levels of phosphorylated form of $p185^{HER2/neu}$. Normally, increases in the active phosphorylated form of $p185^{HER2/neu}$ induce the PI3-K/Akt pathway that affects an increase in phosphorylated levels of Akt and results in an anti-apoptotic effect (2). On the other hand, activation of the Erk pathway can induce apoptosis in cells exposed to cisplatin (51). Thus, with cisplatin, the combination of an increase in Akt phosphorylation and a decrease in Erk phosphorylation likely prevent an increase in the cytotoxicity by offsetting the apoptotic effects of wild-type p53. With the reduced or minimal effects of DACH-acetato-Pt on phosphorylated $p185^{HER2/neu}$, Akt and ERK, it is likely that functional activation of $p185^{HER2/neu}$ is subdued, which may enable the effect of wild-type p53 to predominate and result in increased cytotoxicity.

In conclusion, our study has demonstrated that HER2/neu overexpression differentially affects the function of activated wild-type p53 depending on the agent that is responsible for p53 induction. It is likely that the differential effect is a result of either 1) the qualitative difference in post-translational modification of p53 affected by independent signaling pathways that are activated by cisplatin and DACH-acetato-Pt, 2) the quantitative difference in the degree of activation of the $p185^{HER2/neu}$ pathway or 3) both. The findings support our hypothesis that introduction of wild-type p53 in combination with the analog could be an effective treatment against cells overexpressing HER2/neu.

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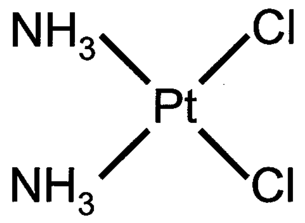
FIGURE LEGENDS

Figure 1 Structures of cisplatin and DACH-acetato-Pt.

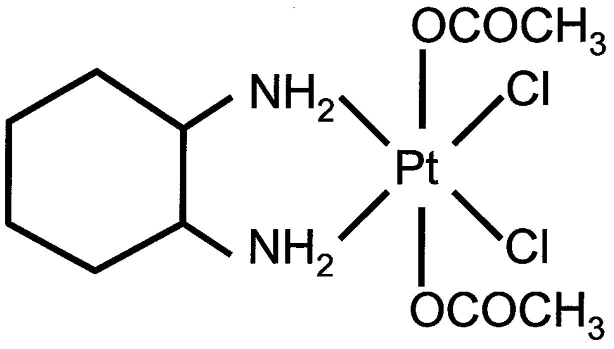
Figure 2 Western immunoblot analysis. A. Basal levels of p185$^{HER2/neu}$ and p53 in SK-OV-3/neo and TS clones at 37°C. MCF-7 cells were used as positive control for p53 and negative control for p185$^{HER2/neu}$. B. Basal levels of p53 and p21 in SK-OV-3/neo and TS clones cultured at 37°C or 32°C for 24 hours. Basal levels of the active phosphorylated p185$^{HER2/neu}$ or the phosphorylated Akt did not change by lowering the incubation temperature to 32°C (data not shown).

Figure 3. Time course of induction of proteins in the TS4 clone treated with 5 x IC_{50} concentration of cisplatin (A) or DACH-acetato-Pt (B) (29.3 μM cisplatin or 29.6 μM DACH-acetato-Pt) at 32°C. The figure indicates results following Western blot analysis.

Figure 4. Quantitation of bands observed following Western immunoblot analysis. The bands observed in Figure 3 were quantified by laser densitometry and plotted over time (• cisplatin; o DACH-acetato-Pt). The serine 392 phosphorylated-p53 is not shown, as it was not detectable in untreated TS4 cells or after treatment with DACH-acetato-Pt.


NH$_3$
Cl
Pt
NH$_3$
Cl

Cisplatin


OCOCH$_3$
NH$_2$
Cl
Pt
NH$_2$
Cl
OCOCH$_3$

1R,2R-DACH-$(Ac)_2Cl_2$-Pt(IV)

Figure 1

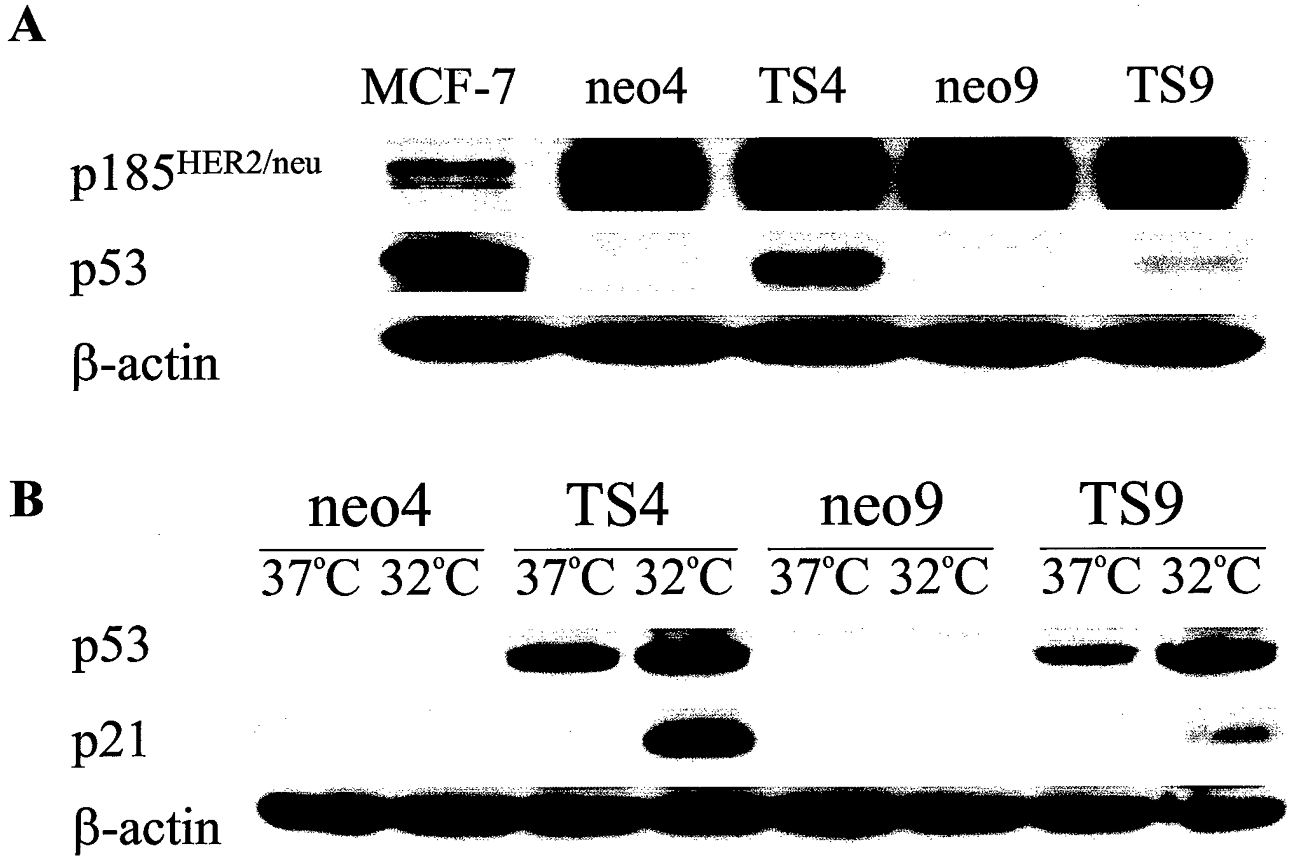
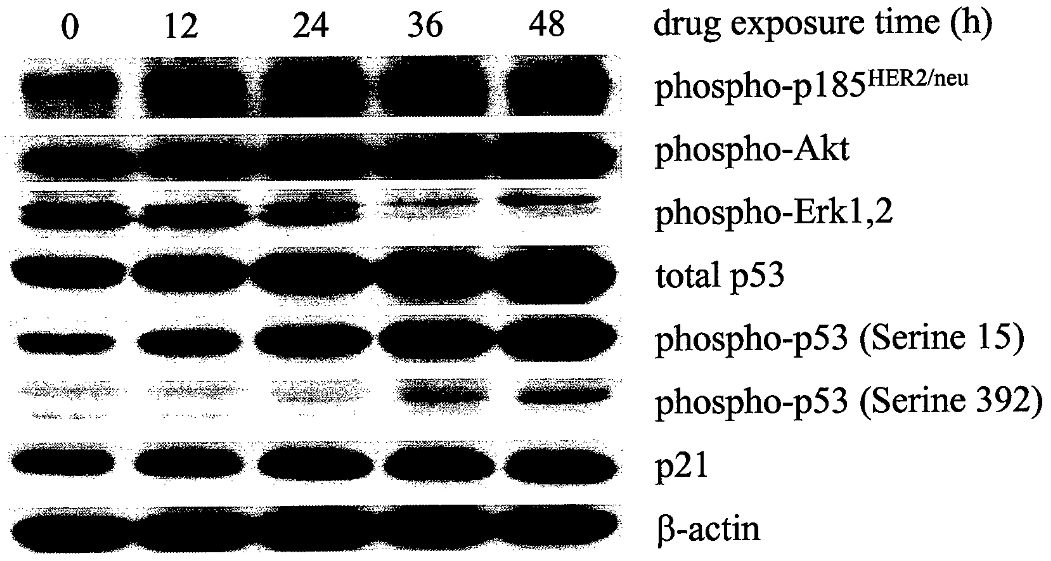

A
MCF-7
neo4
TS4
neo9
TS9
p185HER2/neu
p53
β-actin
B
neo4
TS4
neo9
TS9
37°C 32°C
37°C 32°C
37°C 32°C
37°C 32°C
p53
p21
β-actin

Figure 2

A. cisplatin


0
12
24
36
48
drug exposure time (h)
phospho-p185HER2/neu
phospho-Akt
phospho-Erk1,2
total p53
phospho-p53 (Serine 15)
phospho-p53 (Serine 392)
p21
β-actin

B. DACH-acetato-Pt

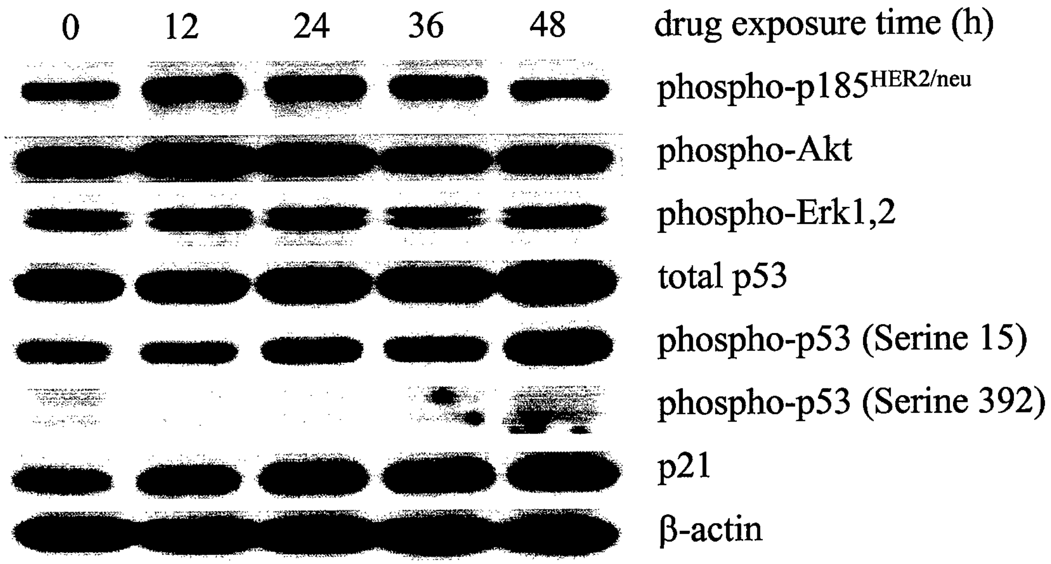

0
12
24
36
48
drug exposure time (h)
phospho-p185HER2/neu
phospho-Akt
phospho-Erk1,2
total p53
phospho-p53 (Serine 15)
phospho-p53 (Serine 392)
p21
β-actin

Figure 3

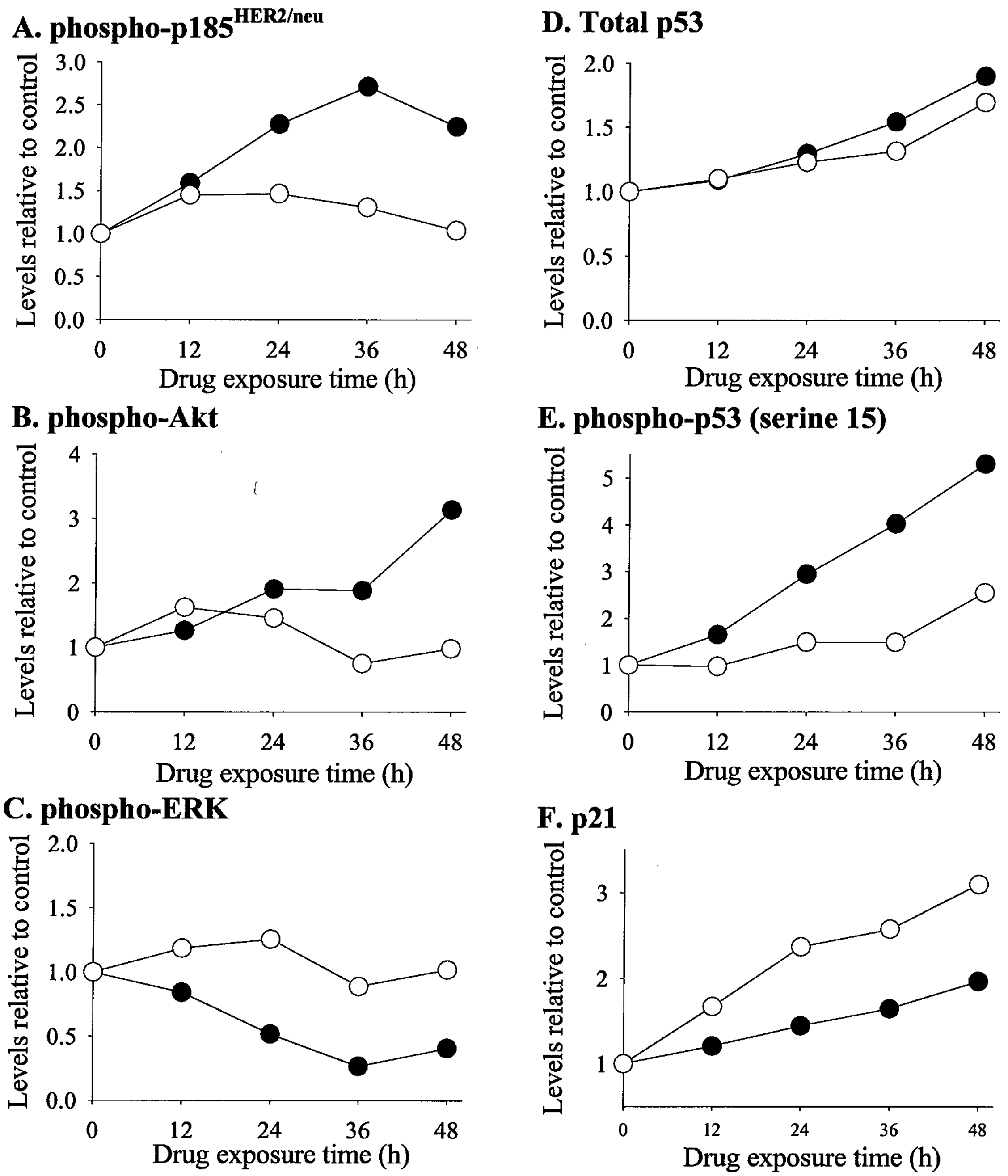

A. phospho-p185$^{HER2/neu}$
Levels relative to control
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
12
24
36
48
Drug exposure time (h)
B. phospho-Akt
Levels relative to control
4
3
2
1
0
0
12
24
36
48
Drug exposure time (h)
C. phospho-ERK
Levels relative to control
2.0
1.5
1.0
0.5
0.0
0
12
24
36
48
Drug exposure time (h)
D. Total p53
Levels relative to control
2.0
1.5
1.0
0.5
0.0
0
12
24
36
48
Drug exposure time (h)
E. phospho-p53 (serine 15)
Levels relative to control
5
4
3
2
1
0
0
12
24
36
48
Drug exposure time (h)
F. p21
Levels relative to control
3
2
1
0
12
24
36
48
Drug exposure time (h)

Figure 4

Table 1. Effect of TS p53 transfection on the cytotoxicity of SK-OV3 cell line

	37 degrees		32 degrees	
clone	cisplatin	DACH-acetato-Pt	cisplatin	DACH-acetato-Pt
neo4	0.84 ± 0.24*	1.58 ± 0.29	5.94 ± 1.08	14.3 ± 3.1
TS4	1.63 ± 0.12**	2.19 ± 0.39	5.86 ± 0.82	5.91 ± 1.35**
TS4/neo4 ratio	*1.94*	*1.39*	*0.99*	*0.41*
neo9	2.33 ± 0.61	11.1 ± 1.5	4.27 ± 0.82	22.7 ± 5.6
TS9	2.16 ± 0.40	8.78 ± 2.43	3.55 ± 1.76	4.63 ± 0.24**
TS9/neo9 ratio	*0.93*	*0.79*	*0.83*	*0.20*

*Mean ± SD; $^{**}P<0.05$, vs. neo with *t*-test; n=3

Table 2. Effect of TS p53 transfection on the cytotoxicity of SK-OV3 cell Boichemical pharmacology of cisplatin and DACH-acetato-Pt in SK-OV3/clone4

	37 degrees		32 degrees	
clone	cisplatin	DACH-acetato-Pt	cisplatin	DACH-acetato-Pt
Platinum uptake (ng Pt/mg protein)				
neo4	81.8 ± 33.8*	31.8 ± 12.1	46.4 ± 21.4	19.2 ± 5.4
TS4	81.5 ± 32.3	31.8 ± 13.7	45.1 ± 21.1	19.0 ± 6.8
DNA adducts (ng Pt/mg DNA)				
neo4	39.8 ± 12.8	7.7 ± 1.4	20.8 ± 10.7	7.9 ± 2.5
TS4	30.1 ± 10.6	7.1 ± 2.1	19.7 ± 13.4	8.6 ± 3.2
DNA damage tolerance (ng Pt/mg DNA at IC_{50})				
neo4	0.33 ± 0.09	0.12 ± 0.02	1.24 ± 0.22	1.13 ± 0.24
TS4	0.49 ± 0.03**	0.16 ± 0.03	1.15 ± 0.16	0.51 ± 0.12**

*Mean ± SD; **P<0.05, vs. neo4 with *t*-test, n=3-4